External Validation of Consensus Molecular Subtyping of Colorectal Adenocarcinoma

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Abstract

Background: Colorectal cancer (CRC) is the second most diagnosed cancer in New Zealand, and New Zealand has amongst the highest rates per capita of CRC in the world. CRC is a highly heterogeneous disease with varying clinical outcomes, morphology and treatment response. Various molecular classifications have been previously described with varying degrees of success in prognostication and predicting response to treatment, but none has been successful in establishing tailored treatments based on molecular profiling. In 2015, a large international consortium published a new classification system based on gene expression data. While this classification system shows considerable promise in subtyping CRC, it has yet to be adopted widely. We aim to validate this Consensus Molecular Subtype (CMS) classification system using CRC stored within the Cancer Society Tissue Bank (CSTB).

Method: More than 300 snap-frozen tumour tissue samples were available from the CSTB between 2002 to 2012. RNA was extracted from 20 milligram of tumour samples and sequenced using the Illumina HiSeq platform. Raw sequence reads were checked and mapped to human reference genome. Gene expression were quantified based on the number of reads mapped to particular gene loci. Gene Expression profiles from each patient was used as input data to the publicly available CRC subtype classifier from the Colorectal Cancer Subtyping Consortium (CRCSC) and subclassified into four individual subtypes. The clinicopathological, treatment, outcome and 5-year follow-up data were collected retrospectively from patient notes.

Results: Of the 306 patients, 19.3% were CMS1, 45.4% were CMS2, 13.1% were CMS3 and 5.2% were CMS4. 17% of CRCs were not classifiable. CMS1 tumours were mainly right-sided, node-negative, poorly-differentiated. CMS2 tumours

were predominantly left-sided tumours found in male patients and were mainly Microsatellite stable (MSS). CMS4 tumours were mainly found in younger patients with left sided tumours and present at an advanced stage. The five-year survival rates for patients with CMS1, CMS2, CMS3 and CMS4 tumours were 74.6%, 71.2%, 67.5% and 43.8% respectively (P=0.03). There was no significant difference in the chemo-response rate between the four subtypes. When subtyping of hepatic metastasis was looked at 50% had incongruent classification. 75% of these patients received neoadjuvant therapy prior to hepatic resection (P = 0.02).

Conclusion: The CMS classification is reproducible on a large scale and showed distinct clinical and histological features within each subtype. Further clinical studies are required to assess responsiveness of each subtype to adjuvant therapy and targeted therapy and the subtype congruency in distant metastasis.

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List of Abbreviation

ACPS	Australian Clinico-Pathological Staging
AJCC	American Joint Committee on Cancer
APC	Adenomatosis Polyposis Coli
ATP	Adenosine Triphosphate
BCL-2	B Cell Lymphoma 2
CDK	Cyclin Dependant Kinase
CIMP	CpG Island Methylator Phenotype
CIN	Chromosomal Instability
CK1α	Casein Kinase 1 Alpha
CMS	Consensus Molecular Subtypes
CRC	Colorectal Cancer
CRCSC	CRC Subtyping Consortium
CSTB	Cancer Society Tissue Bank
CT	Computed Tomography
DFS	Disease Free Survival
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EMT	Epithelial-Mesenchymal Transition
EVI	Extramural Venous Involvement
FDA	United States Food and Drug Administration
GDP	Guanosine diphosphate
GSK3	Glycogen Synthase Kinase 3
GTP	Guanosine triphosphate
FZ	Frizzled receptor
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
IED	Isolated Extramural Deposit
IHC	Immunohistochemistry
LR	Local Recurrence
LVI	Lymphovascular Invasion

MAPK	Mitogen activating protein kinase
MAPKK	MAPK Kinase
MAPKKK	MAPK Kinase Kinase
MHC	Major histocompatibility complex
MMR	Mismatch repair
MSI	Microsatellite instability
MSS	Microsatellite stable
N+ve	Node positve
N-ve	Node negative
NGS	Next Generation Sequencing
NZGL	New Zealand Genomics Limited.
OS	Overall Survival
PD-1	Program Cell Death Protein 1
PI3K	Phosphatidylinositide 3-Kinases
PNI	Perineural involvement
PP2A	Protein Phosphatase 2A
RFS	Relapse free survival
RNA	Ribonucleic acid
RTK	Receptor Tyrosine Kinase
SAR	Survival After Relapse
SEER	Surveillance, Epidemiology, and End Results Program
TAM	Tumour Associated Macrophage
TCGA	The Cancer Genome Atlas
TGF-β	Transforming Growth Factor Beta
TNM	Tumour, Nodal, Metastases staging
UICC	Union for International Cancer Control
WHO	World Health Organization

1. Background

1.1 Introduction

Colorectal cancer (CRC) is the second most diagnosed cancer in New Zealand with over 3000 new cases diagnosed annually[1], and New Zealand has amongst the highest rates per capita of colorectal cancer in the world, with a median annual age standardised rate per 100,000 for males of 55.2 (range, 50.8–56.2) and for females of 44.1 (range, 42.5–45.0) [2, 3]. CRC has been traditionally approached as a single disease while in reality it is a highly heterogeneous and complex disease. The Tumour, Node, Metastasis (TNM) staging[4] and histological grading systems[5] have traditionally been the main tools used for staging, classifying and guiding the use of surgery and adjunctive therapy for treating CRC. Complete surgical excision of the primary malignancy coupled with adjuvant chemotherapy for high risk patients is the only curative option for treating CRC[6]. Despite this, survival and relapse rate vary considerably in CRC tumours with similar histopathological features[7-10]. To overcome this issue, significant efforts have been made to better understand the limitations of TNM staging, the underlying genetics of CRC, and the heterogeneity of CRC in order to develop novel classification system to prognosticate and to predict the clinical behaviour more accurately.

1.2 Traditional TNM staging and World Health Organization (WHO) grading

Traditionally CRCs were staged and classified by the Dukes, Australian Clinico-Pathological Staging (ACPS) or the TNM system. Cuthbert Duke first published a staging system for rectal cancer in 1932 through the analysis of the depth of invasion and presence of locoregional lymph node involvement[11]. This is then staged into Dukes' A, B and C (Table 1). It provided reliable prognostication for

CRCs but fails to consider patients who had metastasis or incurable disease. Since then multiple modifications were made by the likes of Kirklin, Dockerty, Waugh[12] and Astler-Coller[13] (see Appendix). However, these modifications prove to be complex and not easily remembered. In 1981 the ACPS system (Table 2) was proposed at the gastrointestinal congress in Brisbane, Australia[14]. This was later validated by the Concord Hospital, Sydney in 1984[14]. The ACPS is commonly used in Australia and the key is the addition of stage 'D' which denotes the presence of distal metastasis or un-resectable, locally advance disease.

The TNM staging system, which is more universally used, was first devised between 1943 and 1952 to stage solid tumours based on the extent of the tumour (T), lymphatic and nodal involvement (N) and presence of systemic metastasis (M)[4]. Since then the Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC) have contributed to its further development to maintain global standards for classifying the extent of tumour spread. This system provided a more accurate and detailed analysis CRCs. Table 3 to 6 shows the TNM staging in detail and Table 7 shows the survival differences between the different stages in CRC[15].

CRC can be histologically classified into mucinous adenocarcinoma (If >50% of the lesion consist of mucin), Signet-ring cell adenocarcinoma (When >50% of cells contain prominent intracytoplasmic mucin), adenosquamous carcinoma, medullary adenocarcinoma and undifferentiated adenocarcinoma[5]. Histological grading of CRC is based on the percentage of the tumour showing gland like structure and is divided into well (> 95% gland like structure present), moderate (50-95% gland like structure present) and poorly differentiated (< 50% glandular structure present) CRC[5].

Table 1: Dukes' Classification of Rectal Cancer [11]

Dukes’	
A	Carcinoma limited to the wall of the rectum
B	spread by direct continuity to the extra-rectal tissues but has not yet invaded the regional nodes
C	metastases are present in the regional lymph nodes

Table 2: ACPS Staging of CRCs [14]

ACPS	
A	Tumour infiltrates muscularis propria
B	Tumour infiltrates beyond muscularis propria
C	Local nodal involvement
D	Tumour transected or distal metastasis

Table 3: AJCC T-Staging of CRCs [4]

T staging	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in-situ. Intraepithelial or invasion of lamina propria
T1	Tumour invades submucosa
T2	Tumour invade muscularis propria
T3	Tumour invades through mascularis propria and into pericolorectal tissue
T4a	Tumour penetrates to the surface of visceral pritoneum
T4b	Tumour directly invades or is adherent to other organs or structures

Table 4: AJCC N-Staging of CRCs [4]

N Staging		
Nx		Regional nodes cannot be assessed
N0		No regional lymph node metastasis
N1		Metastasis in 1-3 regional lymph node
	1a	Metastasis in one regional lymph node
	1b	Metastasis in 2-3 regional lymph node
	1c	Tumour deposit(s) in subserosa, mesentery, non-peritonealised pericolorectal tissue without regioal lymph node involvement
N2		Metastasis in 4 or more regional lymph node
	2a	Metastasis in 4-6 regional lymph node

	2b	Metastasis in 7 or more regional lymph node

Table 5: AJCC M-Staging of CRCs [4]

M staging		
Mx		Distant metastasis cannot be assessed
M0		No distant metastasis
M1		Distant metastasis
	1a	Metastasis confined to one organ or tissue
	1b	Metastasis more than one organ site or peritoneum

Table 6: AJCC Overall TNM Staging for CRCs [4]

Overall staging				
Stage		T	N	M
0		Tis	N0	M0
I		T1/2	N0	M0
II	A	T3	N0	M0
	B	T4a	N0	M0
	C	T4b	N0	M0
III	A	T1/2	N1	M0
		T1	N2a	M0
	B	T3-4a	N1	M0
		T2-3	N2a	M0
		T1/2	N2b	M0
	C	T4a	N2a	M0
		T3-4a	N2b	M0
		T4b	N1/2	M0
IV	A	Any T	Any N	M1a
	B	Any T	Any N	M1b

Table 7: SEER Five-Year Survival Based on Different Staging [11]

5-year relative survival (%)			
Year of diagnosis	Total	Males	Females
1975-1977	50.6	50.1	51.1
1978-1980	52.3	51.3	53.2
1981-1983	55.2	55.6	54.8
1984-1986	58.3	58.8	57.8
1987-1989	60.2	60.7	59.6
1990-1992	62.0	62.2	61.7
1993-1995	59.9	60.1	59.6

1996-1998	52.2	62.4	62.0
1999-2002	64.9	65.7	64.2
2003-2009	65.4	65.9	65.0
2009	64.8	65.9	65.0
Stage			
All	64.2	64.7	63.8
localised	91.4	91.7	91.0
Regional	70.8	70.6	70.9
Distant	12.3	11.9	12.6
Unstaged	26.5	31.0	23.1

Although the combination of the histological appearance, grading and the staging of CRC have provided a widely-accepted tool for prognostication and predictive value, it fails to consider the underlying differences in genetics and, molecular signalling pathway and environmental impact. Thus, it serves as a poor tool in prognosticating CRC tumours with similar histopathological features, grading and stage. Relapse and survival rates vary widely between studies[7-10], some having a relapse rate as high as 60% within the first two years of resection[16]. Deciding which patients are most at risk of relapse regardless of stage of disease and tailoring appropriate treatment is of upmost importance rather than relying on traditional therapeutic recommendations[6].

1.3 CRC genetics, and molecular signalling pathway

Traditionally three molecular pathways have been described that lead to phenotypically distinct CRC (Figure 1): chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP)[17]. In 2012 The Cancer Genome Atlas (TCGA) published their genome wide analysis of CRC[18]. It showed not only the 32 commonly recurring mutations and its prevalence, but more importantly it showed the five-key

signalling pathways involved in the tumorigenesis of CRC (WNT, MAPK, PI3K, TGF-β and P53 pathways) within the CIN pathway and its prevalence.

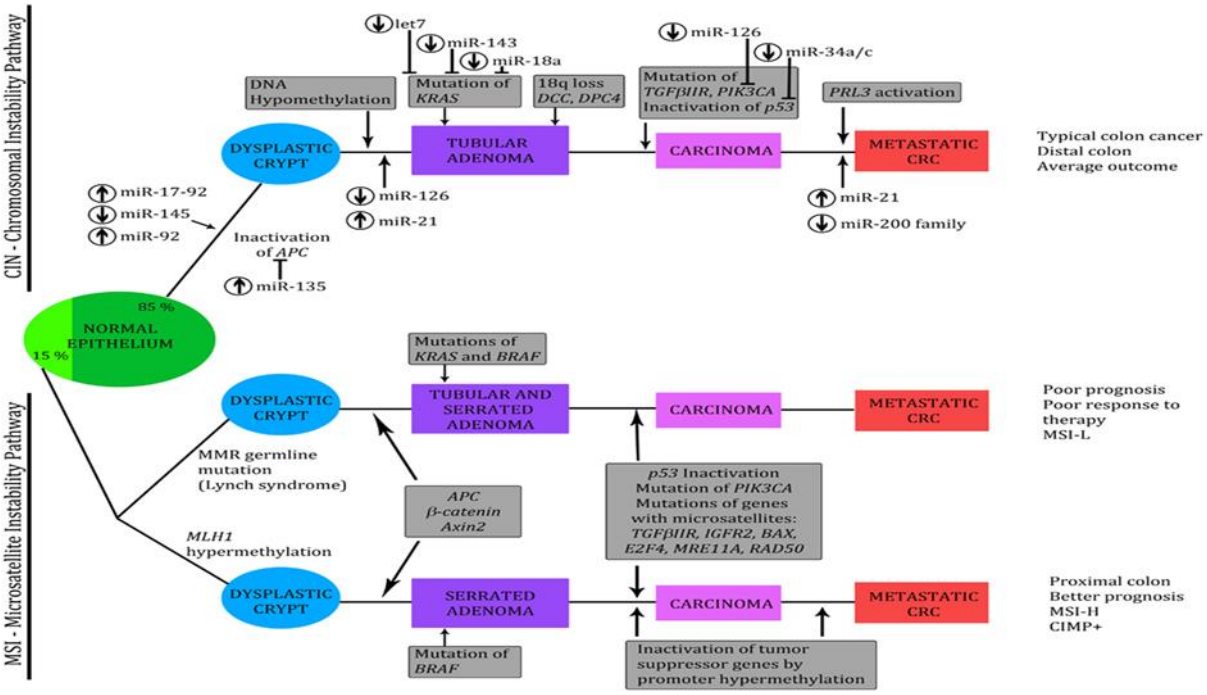

Figure 1 Genetic model of the adenoma to carcinoma sequence.

Canonical WNT pathway – Canonical WNT signalling pathway (WNT/β-catenin pathway) is a signalling pathway that leads to the accumulation of β-catenin that translocates into the nucleus to act as a transcriptional coactivator. This leads to subsequent cellular growth and proliferation[20]. In the absence of WNT, β-catenin is protealysed and degraded by a degradation complex consisting of axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 (GSK3) and casein kinase 1α (CK1α)[21]. When WNT binds to its receptor, frizzled (FZ), It causes destabilisation of the degradation complex (dissociation of Axin, APC and GSK3) leading to an accumulation of β-catenin and subsequent transcriptional upregulation of genes[22]. Disruption of the canonical WNT pathway has been reported in 93% of all CRC and the genes

commonly affected in this pathway are *APC* (70% of all sporadic CRCs), *CTNNB1* (β-catenin gene - 80%)[18, 23, 24]. Other mutations include the *SOX9* mutation and upregulation of *FZ* (17%)[18].

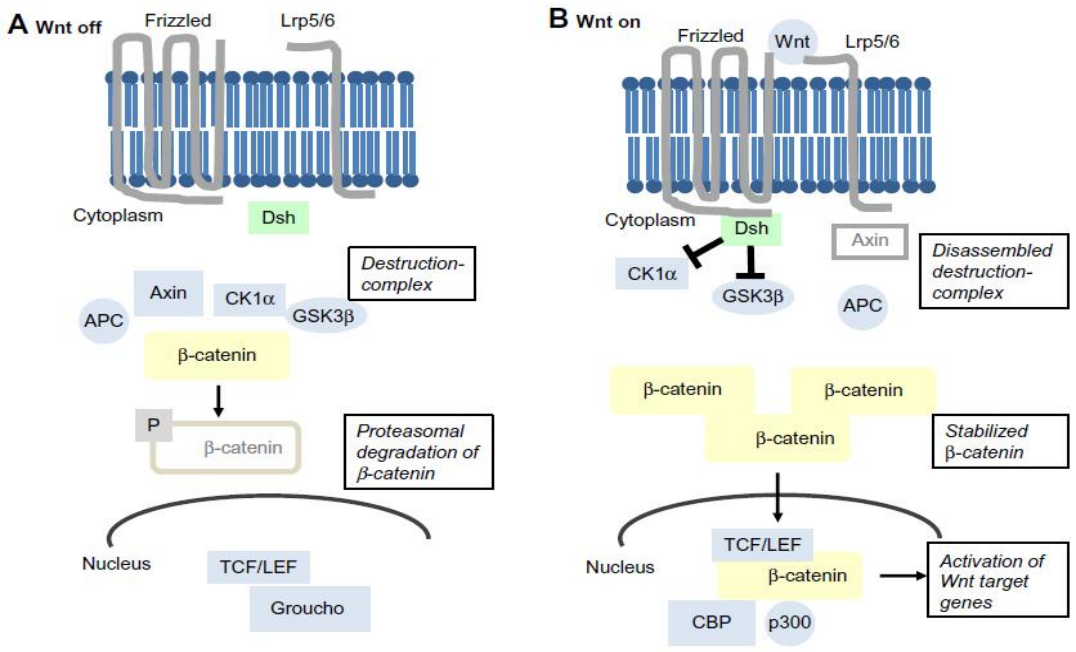

Figure 2. Conical WNT signalling pathway

MAPK signalling pathway – Mitogen activating protein kinase (MAPK) signalling pathway consists of highly conservative family of protein kinases which exist in an inactivated form and is responsible for the transduction of signal from a surface receptor to the Deoxyribonucleic acid (DNA). The MAPK pathway consists mainly of a receptor tyrosine kinase and three subsequent protein kinases; MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK. When an extracellular signal such as growth factor binds to its receptor, the receptor tyrosine kinase (RTK) gets activated. It subsequently phosphorylates and activates downstream MAPKKK (e.g. BRAF, CRAF). MAPKKK then phosphorylate and activates MAPKK (e.g. MEK1, MEK2, MEK5) which then activates MAPK (e.g. Erk1, Erk2, JNK1). MAPK activates specific MAPK-activated protein kinases (MAPKAPK) which leads to a series of cellular changes

such as growth, differentiation and development[26]. RAS/MEK/Erk pathway is one of the more well-known MAPK signalling pathway. Upon binding of extracellular growth factor (e.g. Epidermal Growth Factor, Insulin-like Growth Factor, Human Epidermal Growth Factor 2) to its receptor, RTK activates intracellular RAS by swapping a GDP for a GTP[27]. Three RAS genes have been implicated with tumorigenesis (*KRAS, HRAS and NRAS*). The activated RAS then activates the first of the three protein kinases, RAF (MAPKKK) through phosphorylation. RAF subsequently phosphorylates downstream MEK (MAPKK) and Erk (MAPK). When Erk gets activated, it phosphorylates a whole series of downstream proteins (MAPKAPK) which leads to the activation of transcription factors, thus allowing proliferation and cellular growth to occur[28]. This pathway is commonly dysregulated in CRCs with *KRAS* mutation noted in 35%, *BRAF* mutation in 10% of CRC[18]. Other mutated genes within this pathway includes *NRAS, ERBB. KRAS, NRAS* and *BRAF* mutations have been shown to be mutually exclusive[29, 30].

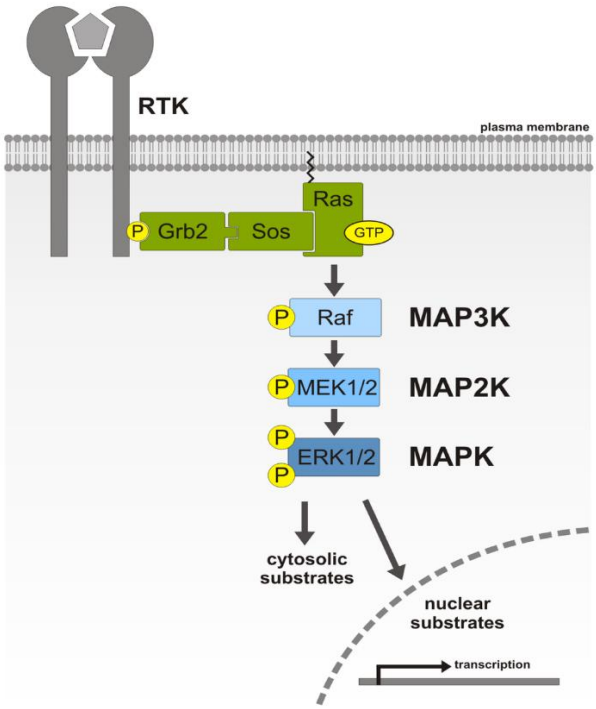

Figure 3. MAPK signalling pathway

PI3K/ATK/mTOR pathway – This intracellular signalling pathway is important in the regulation of the cell cycle. Binding of cell-surface receptor by growth factors, e.g. EGF, IGF-1, Insulin, leads to phosphorylation of PI3K, which in turn results in downstream phosphorylation of PIP2 and PIP3[32, 33]. This activates AKT which ultimately leads to the activation of mTOR[24]. mTOR functions as a serine-threonine protein kinase that regulates cell growth, proliferation, motility and division[34]. AKT also inhibits the pro-apoptotic protein BCL-2 and increases degradation of P53 (see P53/P21/CDK pathway), thus preventing apoptosis[24, 35]. This pathway is downregulated and inhibited by PTEN which directly inhibits PI3K[24]. Common genetic aberrations affecting this pathway in CRC includes the inactivation of PTEN (10%), upregulation of AKT (commonly seen in the early stages of CRC) and activating mutation in PI3K[18, 24]

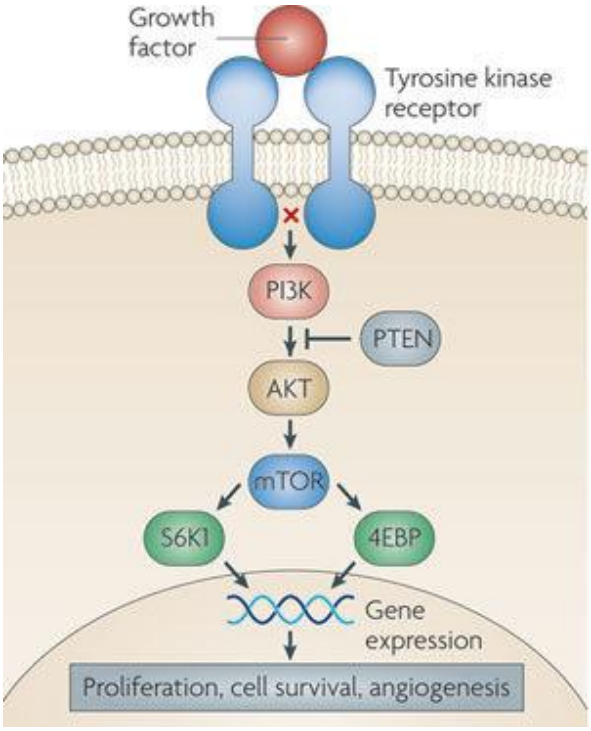

Figure 4. PI3K/ATK/mTOR pathway

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TGF-β pathway – TGF-β has a myriad of function in vivo, functioning as a tumour suppressor, angiogenesis, immunosuppression and regulating cellular

proliferation[37, 38]. Almost all human cells produce TGF-β and have receptors for it[38]. The receptor is a dimer consisting of two subunits (TβRI and TβRII). Upon binding of TGF-β, TβRII is activated causing phosphorylation of TβRI. Consequently, this triggers a wide variety of intracellular signalling pathway; most important in relation to tumorigenesis is the SMAD signalling pathway[37]. Phosphorylation of SMAD 2 and 3 occurs and this binds to SMAD 4 forming a complex that translocates into the nucleus. This leads to downstream transcription of genes, particularly P15, CDK2, CDK4, cyclin A and Cyclin E, leading to cell cycle arrest at the G1 phase[39]. This pathway is dysregulated in 80% of CRCs leading to dysregulation of proliferation, growth and differentiation[18, 24]. Frameshift mutation of the *TβRII* gene occurs in 85% of CRCs, especially with hereditary nonpolyposis colorectal cancer (HNPCC)[40]. SMAD4 is mutated in 30% of CRCs[18].

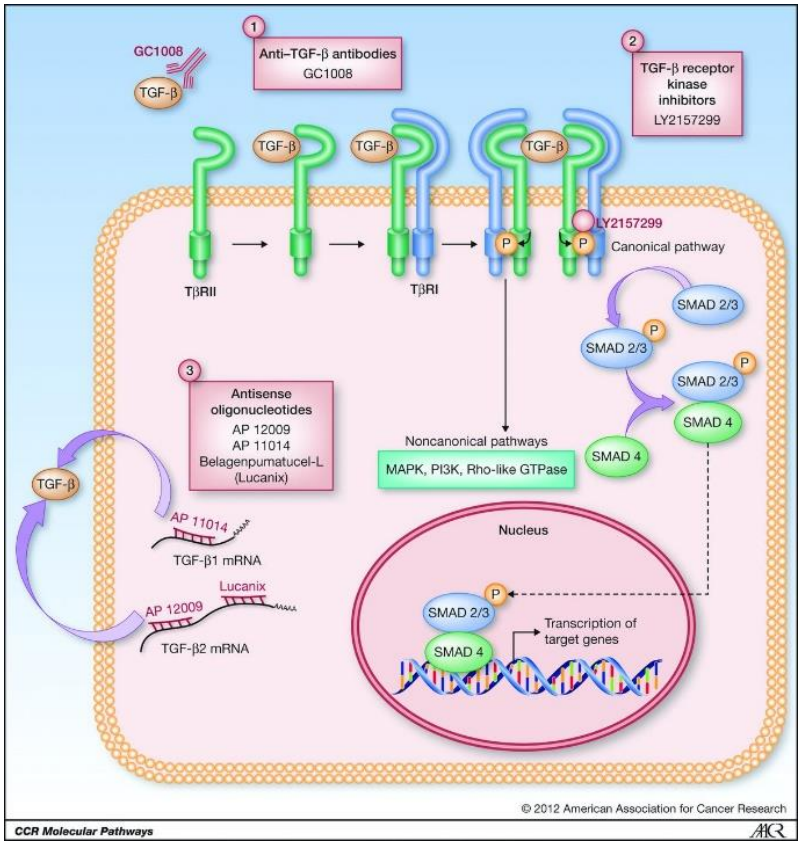

Figure 5. TGF-β pathway

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P53/P21/CDK pathway - This pathway ultimately leads to cell cycle arrest in the G1/S phase, preventing further cellular growth, proliferation and DNA repair, cellular senescence or apoptosis from occurring[42]. The initiation of this pathway starts with the activation of the P53 protein via a stress signal. Once p53 is activated, it binds to DNA and facilitates the transcription of multiple genes including *hsa-miR34a*, p21 and several other downstream genes[42]. *hsa-miR34a* is a tumour suppressor gene and has a pro-apoptotic and senescent properties. It has been shown to be strong inhibitors of BCL-2, cyclin D1 and cyclin E2[43]. P21 binds to CDK complex, thereby inhibiting its action and arresting cell cycle and preventing the progression of cells from G1 to S phase. Upon cell cycle arrest, p53 induces DNA repair, senescence or apoptosis[42].

These signalling pathways are not mutually exclusive and have complex interactions; cross-communication commonly occurs between them. This leads to a wide variety of phenotypical presentation of CRC and account for the inter-tumoral heterogeneity of CRC, which is later discussed.

1.4 Heterogeneity of CRC:

Tumour heterogeneity is collectively caused by intra-tumoral, inter-tumoral differences and the complex relationship and interaction of tumours with its their microenvironment either pre-operatively or perioperatively[44].

Intra-tumoral differences: Each tumour is made up of a multitude of sub-clonal cells, each having distinct biological and molecular properties with varying degrees of mutation[44]. In 1976, Peter Norwell described the concept of clonal structure and evolution of sub-clones in cancer[45]. He states that the clonal relationship among cells arise when selection operates on individual cells to induce a survival advantage or disadvantage. It can also emerge because of

mutation over time without selection (genetic drift). This has since been reported in many epithelial and haematological malignancies. The first study[46] to show such clonal "evolution" was from a lobular breast cancer in which the authors described a substantial number of sub-dominant somatic mutations becoming dominant from the primary tumour to the metastatic population through a process known as clonal expansion over a nine-year period. This has been replicated in CRC whereby there is discordance of mutation between primary and metastatic population as shown in the accumulation of mutation in *KRAS, BRAF* and *PIK3CA*[44]. Todaro et al[47] consolidated this further by stating that CRC starts as a stem cell disease and through clonal evolution, forms sub clones of cancer stem cells that confer survival benefit and chemo-resistance which subsequently leads to recurrence or distal metastasis. The presence of these different sub-clones within each tumour and selection processes leads to the differences in the clinical behaviour, metastatic potential and drug-resistance in phenotypical and histologically similar tumours.

Inter-tumoural differences: Inter-tumoral differences is the difference between tumours within a diagnostic group such as CRC. Different signalling and molecular pathways are involved in the tumorigenesis of CRCs. Traditionally three molecular pathways have been described that lead to phenotypically distinct CRC: chromosomal instability (CIN), microsatellite instability (MSI) and CpG island methylator phenotype (CIMP)[17]. They can occur in combination or can be mutually exclusive, generating inter-tumoural heterogeneity [17, 44]. CIN describes the accumulation of a multitude of mutations in the oncogenes and tumour suppressor genes[44]. It has been commonly conceptualised as the step wise accumulation of mutation particularly the *APC, KRAS, DCC* and *P53* gene[17, 48-50]. It is known that the total accumulation rather than the sequence of mutation and allelic loss that is responsible for tumorigenesis and the tumours biological behaviour[17]. In 1989 Fearon and Volgestein [49] showed that there

was a median of four to five allelic deletion in individual chromosomes in colorectal carcinomas and that patients with greater number of losses had a considerably worse prognosis despite having tumours of similar grade, size and stage.

The CIMP pathway on the other hand follows an entirely different molecular pathway, involving the hyper-methylation of CpG islands within promoter regions which leads to epigenetic silencing of genes, most commonly the *MLH1* gene. These sporadic tumours differ considerably in that they are usually associated with the *BRAF V600E* mutation and clinically develop from sessile serrated adenoma[17]. They are typically poorly-differentiated, right sided malignancies with good overall prognosis[17].

The MSI pathway involves CRCs that arise through the dysfunction of the DNA mismatch repair genes (dMMR genes)[51]. These typically involves the *MLH1, MSH2, MS6* and *PMS2* gene[52]. These gene products are involved in the recognition and repair of mismatches that occur during DNA replication. A defect in this process allows genetic errors and mutations to occur, thereby facilitating the CRC tumorigenesis. These tumours are termed MSI due to the deletion or insertion of repetitive short segments of DNA (also known as microsatellites)[53]. Phenotypically, these tumours are right sided, mucinous, early staged, less likely to metastasise and are typically seen in young or female patients[53].

Host and microenvironment: This is a commonly overlooked source of heterogeneity within CRCs. The phenotypical presentation of CRC can be influenced by a multitude of host and micro-environmental factors [44, 54-58]. The tumour micro-environment is a dynamic network involving the hypoxic microenvironment, tumour cells, stromal matrix, extracellular matrix (ECM), lymphocytes, tumour associated macrophage (TAM), fibroblast and other cells. These have an important role in the initiation, growth, propagation and

metastatic ability of tumour cells. The hypoxic microenvironment within tumour triggers a whole host of metabolic adaptation, favouring growth and propagation of tumour population adapted to the environment. An example of this is via activation of pyruvate kinase isoform M1 and M2 (PKM1/2). Under hypoxic situations, tumours with the tetramer, PKM2, proliferate faster than those with PKM1[54], switching to an anaerobic metabolism to confer a survival benefit; this involves rapid generation of adenosine triphosphate (ATP), biosynthesis of macromolecule and maintenance of appropriate redox state to minimise damage from reactive oxygen species[55]. The hypoxic state also facilitates epithelial-mesenchymal transition (EMT), a key step towards metastasis[55]; hypoxia induces epigenetic modification that represses epithelial genes and activate mesenchymal genes. One such example is the *HDAC3*[56].

Tumour behaviour and histological staging are closely related to the host immune response profile [44, 58]. Immune cells secrete a whole host of cytokines and growth factors that can be either anti-tumoural or pro-neoplastic. The location, quantity and quality of immune cells seen within the tumour also predicts differences with the clinical outcomes[44]. High densities of CD8+, CD45 T cells, TH1 are associated with tumours of earlier stage (I and II) and better prognosis[58]. Whereas TH17 cells are associated with more advance stage and poor prognosis[58]. The same can be said about M2 macrophage, with high levels associated with poor prognosis and high metastatic rates[44]. How these immune contextures are formed and how they evolve throughout the disease process is yet to be fully understood[44].

In addition to this, recent studies into colonic microbiota [59, 60], dietary fibre intake [61], Vitamin C and D [62-64], and sedentary lifestyle [65] have all been shown to contribute to the initiation, progression and metastasis of CRCs.

In summary, the heterogeneity sub clonal population, genetic allelic loss pattern, different signalling and molecular pathway and the "second hit" by environmental and host factors leads to development of genetically and phenotypically distinct tumours with different clinical behaviour and response to treatment. Hence the traditional method of grading and classifying them per histopathological feature is not only crude, but also, it does not take into consideration the molecular behaviour of each individual tumour. Novel molecular based classification systems are thus required to facilitate precision oncological treatment.

1.5 Novel classifications systems:

First attempts at reclassifying CRCs molecularly were done by dividing them based on their MSI, CIN and CIMP status. As previously discussed, these tumours have distinct histological, molecular, phenotypical and clinical differences. Classification of these cancers could be made based on relatively simpler investigations, negating the need for expensive tests such as microarray analysis or high throughput next generation sequencing (NGS). MSI can be diagnosed using NCI consensus panel, analysing five microsatellite markers which includes D2S123, D5S346, D17S250, BAT25 and BAT26[66]. However due to financial constraints, immunohistochemistry (IHC) has been used instead to assess the presence or absence of mismatch repair gene proteins[53]. CIMP can be quantitatively assessed using MethyLight technology®[66]. Many centres have utilised BRAF testing as a surrogate marker for CIMP instead. Refinement to classification based around these markers ultimately led to a classification proposed by Jass et al[67], in which they classify CRCs into 5 types; Type1 being CIMP-high (CIMP-H), Microsatellite instable-high (MSI-H) and *BRAF* mutation, Type 2 being CIMP-H, microsatellite stable (MSS) with *BRAF* mutation, Type 3 being CIMP-low (CIMP-L), Microsatellite stable (MSS) with *KRAS* mutation,

Type 4 being CIN and Type 5 being CIMP-negative and MSI-H. This has led to significant changes in the management of patients with CRCs particularly in terms of screening and follow-up[53].

The downside of this classification is that testing for *dMMR* and MSI is done based on patients who clinically fulfilled the Amsterdam II[68] and revised Bethesda Criteria[69] (See appendix). There are concerns that significant proportions of the patients with MSI, particularly Asian populations, may not be tested as these criteria were based on a western population[70, 71]. Neither IHC nor MSI panel testing has 100% accuracy[53]. With regards to IHC, the biggest barrier to accuracy and interpretation of the test is with regards to uniformity of tissue fixation with formalin, which can lead to patchiness, reduced staining or in some cases complete loss of staining[53]. This classification only identifies 15% of sporadic tumours as MSI tumours[53]. It does not address the differences and heterogeneity in CIN tumours which comprise 85% of all sporadic CRCs. More importantly for clinicians, this classification does not offer a clear prognostication between the five subtypes.

To overcome these issues and building on the current knowledge of the molecular genetics of CRCs, novel strategies such as microarray analysis[72] and high throughput genomic analysis[73-80] have been used to analyse gene expression profiles and classify CRC into molecularly and genetically distinct groups to help better predict, prognosticate and aid the treatment of CRC.

Microarray analysis:

Microarray analysis uses a collection of RNA/DNA probes to analyse and quantify a set of pre-chosen target RNAs. It provides a rapid, easy to use and comparatively less labour-intensive way of analysing and profiling CRCs compared to high throughput genomic analysis. Currently only two commercially available, FDA approved CRC microarray analysis exist – the 18

gene Coloprint® and the 12 gene Oncotype DX®. While these tests have been externally validated in multiple studies [72, 81-83], only the prognostic value of these tests have been validated. Using multivariate analysis, Maak et al showed that Coloprint® was the only independent variable to prognosticate survival and recurrence for stage II CRC[81]. It remains to be seen whether patients offered these tests have improved outcomes over those who are not offered these tests. Microarray analysis also suffers from inherent design bias and are only as good as the genes selected for analysis. It lacks the ability to analyse and quantify the entire transcriptome.

High-throughput genomic analysis:

High-throughput genomic analysis or NGS, has made great strides since its introduction more than a decade ago[84]. With cost reduction and improved sequencing, it allows researchers the ability to carry out a more detailed analysis of the transcriptome and interrogate the entire transcriptome without any prior knowledge of it, thereby eliminating any potential design bias[84]. Ever since TCGA published their genomics analysis of CRC, multiple investigators have attempted to use NGS to reclassify CRCs based on their molecular-genetics. Below is a summary of the six most relevant large-scale studies on this.

Budiska et al[75]

Using unsupervised clustering and NGS, 1113 CRCs were classified into five different gene expression subtypes: surface crypt-like, lower crypt-like, CIMP-H-like, mesenchymal and mixed. CIMP-H-like had similar properties to what was described by the TCGA as being hypermutated tumour (*BRAF*, MSI CIMP-H) with better recurrence free survival (RFS) but poor survival after relapse (SAR). Tumours described as mesenchymal tumours also displayed lower overall survival (OS) and early relapse and was also found to have high levels of epithelial-mesenchymal transition (EMT) related gene expression and resulting

in high rates of distant metastasis. Crypt-like tumours on the other hand had better OS. No distinct histological pattern was observed in any one subtype.

Marisa et al.[76]

In this study, the authors sub-classified CRCs into six subtypes (C1-C6) through the analysis of transcriptomic data of 566 CRCs. C2,3 and 4 were more frequently CIMP-H, showed highest rate of EMT gene expression and tended to be proximal tumours with serrated phenotype. C4 subtype had the poorest OS and RFS. C1,5 and 6 on the other hand were more frequently CIN, CIMP-negative, *TP53* negative and were clinically more distal.

Roepman et al.[77]

By using unsupervised clustering of whole transcriptome data of 188 CRCs, three major groups were identified in this study. This was then further validated with 543 stage II to III CRC tumours. Type A (MMR deficient epithelial subtype) was associated with *BRAF* or *KRAS* mutation, had a higher rate of gene mutation and had a high proportion of MSI. Clinically it was associated with right-sided tumours, female gender, poor differentiation and generally good OS. Type B (proliferative epithelial subtype) was almost exclusively MSS, showed a low rate of gene mutations and had a high rate of chemo-responsiveness. Type C (mesenchymal subtype) showed high frequency of mesenchymal markers and clinically was associated with the poorest OS with exceptionally poor response to chemotherapy.

De Sousa e Melo et al[78]

Again, using unsupervised clustering of whole transcriptome data of 90 CRCs, CRCs was divided into three key groups: CIN, MSI and CIMP-H. This was then validated with more than 1100 tumours. The first two subgroups have been well described. The third group was CIMP-H but MSS. These tumours had serrated

phenotypes and high levels of EMT gene expression. They also had poorer OS and were resistant to epidermal growth factor receptor (EGFR)-targeted therapy.

Sadanadam et al.[79]

This study included 1290 CRCs and divided them into five different subtypes: stem-like, inflammatory, transit-amplifying, goblet like and enterocyte. The first three subtypes, particularly the stem-like group displayed high EMT expression and had particularly poor OS. The latter two had better disease-free survival (DFS) and the authors suggested that these patients may be spared chemotherapy when the tumours were early staged and localised.

Schickler et al.[80]

After validating publicly available datasets involving 1643 tumours, CRCs were classified into two main groups (type 1 and 2) in this study, which were later further sub classified into five subgroups. Type 1 generally had poor OS and was characterised by high EMT gene expression. This group had a mixture of MSI-H and MSS CRC, whereas Type 2 CRCs were mainly MSS, with epithelial phenotype and good OS.

There are multiple similarities and differences between the six classification systems mentioned above. Most of the similarities are related to MSI and EMT tumours (Table 8). The lack of concordance between these classifications could potentially be explained by differences in the cohorts, data processing, genomic analysis technique and clustering algorithms used[85]. Thus, there has been a poor uptake of these techniques universally. There is a need for a more universal classification that is easily and reliably replicable. In 2015, a large international consortium set about addressing this issue and proposed the consensus molecular subtypes (CMS) of CRC[85].

Table 8: Similarities Between the Six Studies

	CRC subtype	
	Common feature	
Classification publication	**MSI**	**EMT enriched**
Budishka et al.	CIMP-H-like	Mesenchymal
Merisa et al.	C2,3	C4
Roepman et al.	Type A	Type C
De Sousa e Melo et al	Type 2	Type 3
Sadanadam et al	Inflammatory	Stem like
Schickler et al	Type 1	Type 1

1.6 Consensus Molecular Subtypes of CRC:

Due to the differences found between the different classification methods previously described[75-80], the CRC Subtyping consortium (CRCSC) was formed to identify any potential core subtyping patterns. Data were pooled from 18 different databases including the TCGA database. 27 nodes were then generated using 6 different classification systems[75-80]. Using an integrating network-based approach and unsupervised cluster algorithm (Markov Cluster Algorithm), recurring patterns were then identified, and CRCs were reclassified based on these recurring patterns. These are described as CMS 1-4[85].

CMS1: These tumours are typically hyper-mutated, hyper-methylated with low somatic copy number alterations (SCNA). The majority of CMS1 tumours are MSI-H and have defects in the *dMMR* gene. *BRAF* was frequently mutated in this group. Receptor tyrosine kinase (RTK) and mitogen-activated protein kinase (MAPK) pathways were the most commonly activated signalling pathway. Histopathologically they were associated with a high level of TH1 and cytotoxic

T cell infiltration, and commonly had higher histopathological grade. Clinically they were frequently diagnosed in females with right-sided tumours. Despite having a relatively good OS, patients were found to have a poorer SAR.

CMS2: This subgroup contained mainly CIN tumours with high SCNA. They had higher levels of oncogene activation and losses of tumour suppressor gene compared to other CIN tumours. Both WNT and MYC signalling pathways were commonly activated. Clinically, these were mainly left-sided tumours and usually of a more advance stage (III, IV). Despite this, these patients had the best OS compared to the other three subtypes.

CMS3: Tumours in this subgroup were also CIN. However, their SCNA count were lower when compared to CMS2 and 4. They were commonly right-sided tumours. *KRAS* mutation were commonly seen. Overexpression of genes and proteins to increase glycolysis and lactate production was found in this subtype; as a consequence of this, these tumours have prominent metabolic adaptation.

CMS 4: This subgroup contained CIN tumours with histological mesenchymal features. EMT related genes were commonly activated. In addition, this subgroup of CRC also showed gene expression profiles that associated with stromal infiltration and overexpression of extracellular matrix protein. Clinically these tumours are often advanced stage and commonly metastasize. Patients with these tumours tend to have a poorer OS and RFS.

When individual mutations were looked at, no individual event or genetic mutation was limited to a specific subtype. The same can be said about signal transduction cascade and pathways. This emphasizes the poor genotype to phenotype correlation in CRCs. It also illustrates the importance that

classification of CRCs should not be based solely on singular validated biomarkers or on histological features.

Having said that, there are issues with CMS system worth considering. A recent publication by Dunne et al [86] showed that intra-tumoural differences can undermine the accuracy of CMS. They found different regions within the tumour itself, e.g. invasive front, tumour core and lymph node metastasis, harbour distinctively different gene expression. They also shown that individual CRC can potentially be misclassified as CMS4 if tissue for analysis was taken from the invasive front. Recent evidence suggests that the presence of EMT-associated genes seen in CMS4 may reflect upregulated genes from fibroblast and mesenchymal cells present in the background rather than directly from the tumour itself[55, 72, 87, 88]. This together with improvements in techniques involved in nucleic extraction and purification, may lead to less CMS4 or the complete absence of it.

1.7 Conclusion:

Though the CMS classification system shows immense promise in the subtyping of CRC and may allow for subsequent tailored precision-based treatment, it needs to be externally validated with large-scale studies. We in Christchurch are in an excellent position to validate this molecular subtyping system, having access to over 500 CRC tissue samples from the Cancer Society Tissue Bank (CSTB), complete with clinical and outcome data for these patients. We aim to evaluate the demography and clinical outcomes of our patient cohort (Chapter 2), validate the clinical behaviour of each individual subtypes (Chapter 3), validate the prognosis and survival outcomes of each individual subtypes (Chapter 4), evaluate the response of individual subtypes to adjuvant therapy (Chapter 5), and finally assess the subtypes of distant metastasis (Chapter 6). It is

hoped that by externally validating the CMS classification, it will allow for future prospective studies on the predictive utility of this system in guiding adjunctive therapy of CRCs.

2. Epidemiology of Colorectal Cancer from Local Cohort

2.1 Abstract

Aim: To perform an epidemiological study on colorectal cancers (CRC) collected in our tissue bank and to identify any potential risk factors affecting recurrence and survival outcome.

Method: Clinical data was retrospectively collected on all CRC patients who had their tissue stored in the Cancer Society Tissue Bank (CSTB) between 2002 to 2012. Only patients with sporadic, non-hereditary tumours were included. Chi-square and Mann-Whitney test were performed to identify associations between variables. Univariate and multivariate binary logistic regression was used to assess correlation and identify potential risk factor.

Results: 480 patients met inclusion criteria with a median age of 74.3 and an equal male to female distribution. CRCs were predominantly right-sided (47.5%). Rectal tumours only accounted for 15.6%. Right-sided tumours had a higher female prevalence (64.8% $P<0.01$), locally advanced (59.1%) and had features of microsatellite instability (MSI). The overall five-year survival was 63.7% with colonic adenocarcinoma having a significantly better five-year survival compared to rectal adenocarcinoma (66% versus 51.9%). The five-year survival rates for stage I-IV disease were 77%, 74.3%, 52.6% and 20% respectively. Only node positivity, metastatic disease at time of surgery, local recurrence and subsequent development of metastasis were risk factors for poorer survival on multivariate analysis (Odds Ratio of 4.28, 7.69, 16.91 and 28.72 respectively). No histological variables were risk factors for decreased survival.

Conclusion: The epidemiology and survival outcomes for the current cohort are comparable to published results worldwide. No clinical or histological variables other than advanced disease and recurrence were reliably predictive of adverse outcomes. Additional methods such as molecular classification may help further in prognostication.

2.2 Introduction

Adenocarcinoma of the colon and rectum is a highly heterogeneous disease and its incidence is on the rise globally. It has been reported in 2012 that globally 1.36 million were diagnosed with colorectal cancer. It is the second most commonly diagnosed cancer in New Zealand[2]. New Zealand has the highest incidence of CRC as previously mention in section 1.1. With the current classification and treatment options available, reported survival and recurrences vary significantly within the literature. The Consensus Molecular Subtyping (CMS) classification sets about reclassifying CRC based on their molecular genetics using next generation sequencing (NGS) technology. Before external validation of this system can be undertaken, it is important to understand the characteristics of the current cohort on which the external validation would be based on.

2.3 Aims

The aims of this study were to identify

- The demographics of the patients with CRCs
- The clinical and histological characteristics associated with recurrence and survival of patients with CRCs
- potential clinical or histological features that may be used to prognosticate treatment outcomes of CRCs.

2.4 Methods

This study was performed as part of a study looking into the external validation of the CMS classification. A retrospective audit was carried out on all CRCs stored within the CSTB between January 2002 and December 2014. Patients were consented for CRCs to be stored in the CSTB prior to surgery. The specifics on

how the CRC tissues were stored and RNA extracted will be discussed in more detail in Chapter 3. Inclusion criteria for this study included all patients with treatment naïve colorectal cancer above the age of 18, with complete clinical data set. Patients who had prior CRCs, metachronous or synchronous CRCs, hereditary tumours, or neoadjuvant chemotherapy or radiotherapy were excluded from the study (Figure 6). The inclusion and exclusion criteria were set as such to allow accurate interpretation of molecular-genetics and its clinical behaviour when external validation of the CMS classification gets underway. Demographic information, post-operative staging, subsequent recurrence, metastasis and histological data were retrospectively collected from patient notes or electronic notes and entered into a custom-built Microsoft Access (Microsoft, Redmond, Washington, USA) database.

For analysis of association, Chi-square test was used for binary data and t-Test for continuous data. Both univariate and multivariate analysis were performed using Cox regression to assess if any clinical or histological variables could account for the recurrence and survival of patients. Kaplan-Meier survival curves were used to assess survival outcomes between groups. These were performed using SPSS® version 20 (IBM Corp®). A P-value of <0.05 was considered significant.

2.5 Results

Over the thirteen-year period, a total of 563 patients with treatment-naïve colorectal cancer were identified from the CSTB. After inclusion and exclusion criteria were applied, 480 met criteria and were subsequently analysed (Figure 6). A total of eighty-three patients were excluded. Sixty-one patients were excluded as they had either synchronous, metachronous tumours. Eleven patients were excluded as they had adenomas as oppose to adenocarcinomas. A further eleven patients were excluded as these were hereditary tumours.

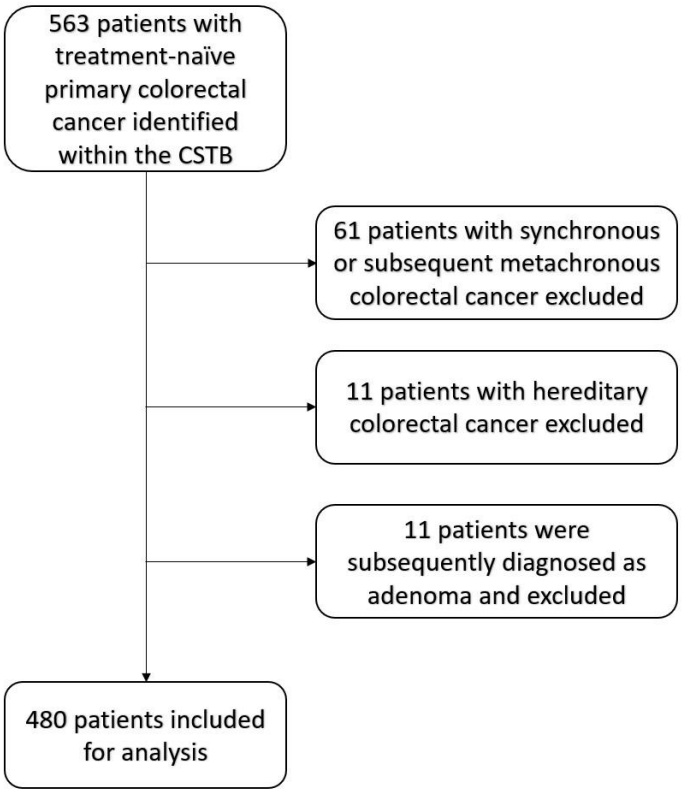

Figure 6 - Flow chart depicting the inclusion and exclusion of patients within this study. CSTB - Cancer Society Tissue Bank

The overall demographics of the patients and the histological features of the CRCs are displayed in Table 9. The median age of presentation was 74.3 years with a range of 28.7 to 94 years. Patients were mainly ethnically European, constituting 97% of the cohort. There was an equal male to female distribution (48.8% vs 51.2% respectively). Right sided tumours were the most predominant at 47.5%. A similar pattern seen when the cohort was broken down into individual age groups; right sided tumours were the most predominant tumours in all age groups (Figure 8). Due to the inclusion criteria, only 15.6% of CRCs were rectal cancers. There were significantly more right sided tumours in females while the opposite could be said for the male cohorts (Table 10). Right sided tumours had a significantly higher proportion poorly-differentiation, and mucinous subtype.

Table 9: Demographics of Patients with CRCs

Total Sporadic Singular Cancer = 480					
	Total	**T1**	**T2**	**T3**	**T4**
n	480	17	87	287	89
Age	74.3	72	74.2	74.3	74.4
Range	28.7 - 94	31.3 - 86.9	36.9-91.5	42.3-94	28.7-92.4
Male	234	10	41	135	48
Female	246	7	46	152	41
Ethnicity					
European	465	17	84	275	89
Asian	4	0	0	4	0
Maori	11	0	3	8	0
Colon	405	15	65	242	83
Right	228	4	32	155	37
Left	177	11	33	87	46
Rectum	75	2	22	45	6
Poorly-Differentiated	100	1	12	59	28
Mucinous	55	1	8	35	11
Signet Cell	4	0	1	3	0
LVI	166	1	15	95	55
PNI	61	0	1	35	25
EVI	70	0	0	39	31
Node Negative	292	13	69	177	33
Node Positive	188	4	18	110	56
Distant Metastasis	30	0	0	15	15
TNM Stage 1	82	13	69	0	0
TNM Stage 2	203	0	0	173	30
TNM Stage 3	165	4	18	99	44
TNM Stage 4	30	0	0	15	15
Patients who had adjuvant treatment	129	2	10	81	36
Node negative	25	0	0	16	7
Node positive	104	2	8	65	29
Subsequent metastasis	81	2	6	47	26
Median Time (days)	437 (34 - 1981)	521 (409 - 634)	1190 (197-1388)	437 (47 - 1981)	369 (34-1098)
Local Recurrence	16	1	0	9	6

Median Time (days)	653 (276-2706)	1933		648 (318 - 2706)	636.5 (276-896)

LVI – Lymphovascular invasion; PNI – Perineural invasion; EVI – Extramural Venous Involvement.

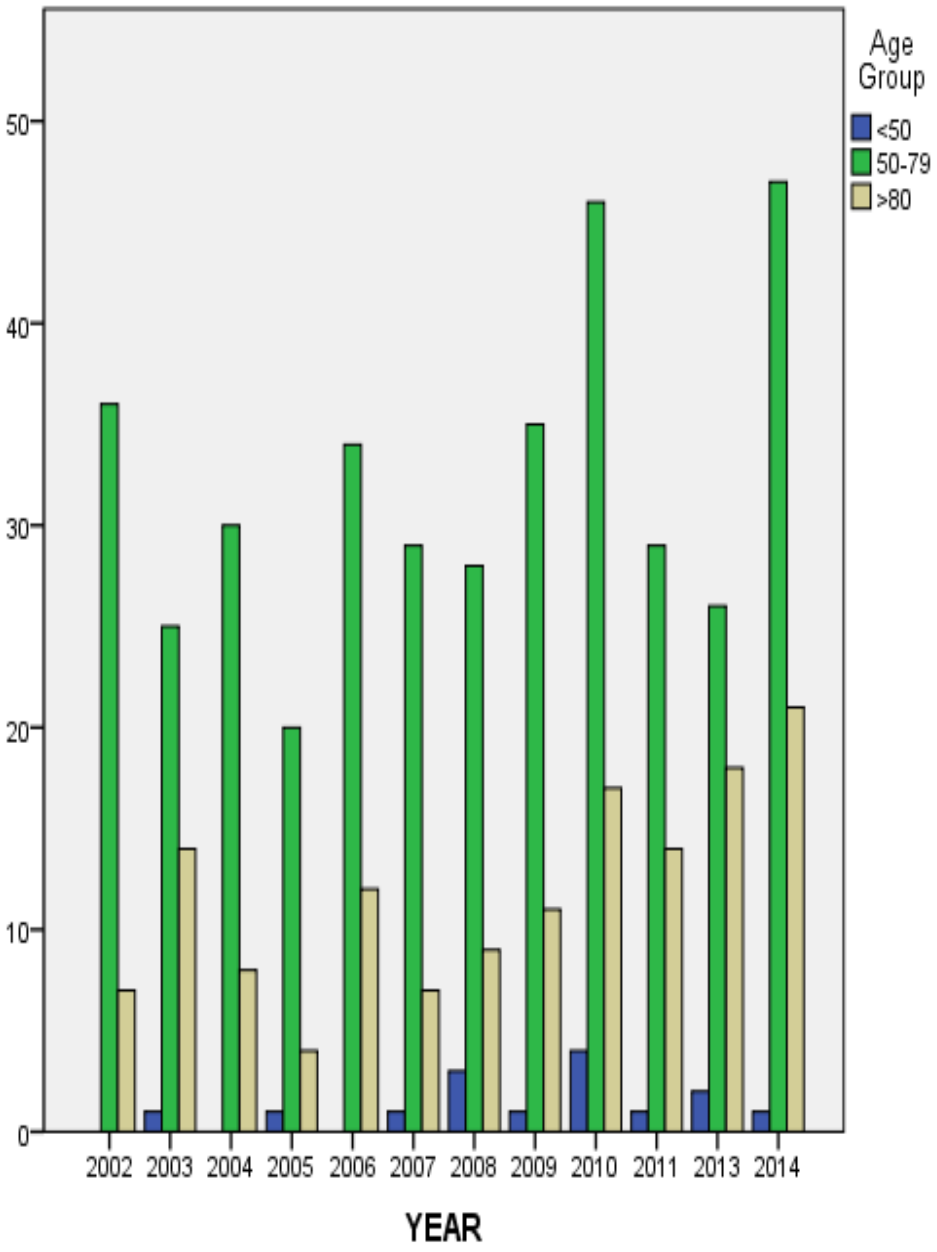

Figure 7. Number of CRC banked per year based on their age group.

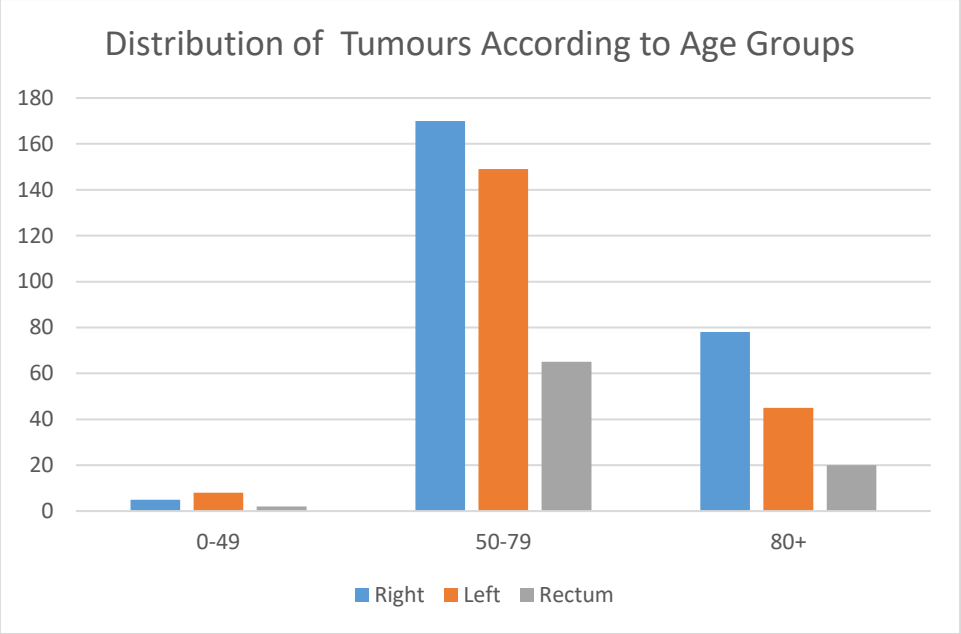

Figure 8. Distribution of CRC based on age groups

Table 10: Comparison of Right and Left Sided CRCs

	Right	Left	OR	P Value
Female	59.1%	42.6%	1.95	<0.01
T>2	84.1%	77.7%	1.51	0.08
N +ve	39.3%	38.1%	0.93	0.79
M+ve	6.0%	7.9%	0.73	0.41
Poor Differentiation	30.2%	13.4%	2.79	<0.01
Mucinous	17.5%	8.4%	2.30	<0.01
Lymphovascular Invasion	37.7%	34.7%	1.14	0.50
Perineural Invasion	11.9%	15.3%	0.74	0.29
Extramural venous invasion	12.3%	20.3%	0.55	0.02
Isolated Extramural Deposit	7.9%	7.4%	1.08	0.84
Local Recurrence	2.8%	4.5%	0.61	0.34
Subsequent metastasis	14.7%	16.8%	0.85	0.53

MSI – Microsatellite instability. N +ve – Node positive. M +ve – Distant metastasis present on diagnosis. Red denotes significant difference between right and left sided CRCs

2.5.1 Analysis of association

There were significant lower survival rates, higher rates of N staging, M staging, subsequent development of metastasis and histological variables, such as poorly differentiated, lympho-vascular invasion (LVI), perineural invasion (PNI) and isolated extramural tumour deposit as the T staging progressively increased. As shown in Table 11, there is an increasing association with these negative clinical variables when the invasion of tumour extends deeper.

Table 11: Clinical and Histological Outcomes of CRCs Based on T-Staging

T staging	1	2	3	4	P-Value
Gender	58.8%	47.1%	47.0%	53.9%	0.56
N+ve	23.5%	20.7%	38.3%	62.9%	<0.01
Stage IV Disease	0.0%	0.0%	5.2%	16.9%	<0.01
Subsequent Distant Metastases	11.8%	6.9%	16.4%	29.2%	<0.01
Poorly Differentiated	5.9%	13.8%	20.6%	31.5%	0.01
LVI	5.9%	17.4%	33.1%	62.5%	<0.01
PNI	0.0%	1.1%	12.2%	28.4%	<0.01
EVI	0.0%	0.0%	13.6%	35.2%	<0.01
IED	0.4%	3.4%	5.6%	15.7%	<0.01
Died from Disease	11.8%	6.9%	15.7%	33.3%	<0.01

N+ve – Node positive disease; LVI – Lymphovascular involvement; PNI – Perineural invasion; EVI – Extramural venous involvement; IED – Isolated extramural deposit;

Patients with clinical features such as male, rectal cancers, nodal positivity, local recurrence, distal metastasis, and histological features, such as poor differentiation, LVI, PNI, extramural venous involvement were associated with higher rates of mortality due to CRC (Table 12). Patients who were male, diagnosed with rectal cancers, node positivity, metastasis at time of surgery, poor differentiation, LVI, PNI, extramural venous involvement, isolated extramural deposits and subsequent local recurrence were also found to have significantly higher rates of subsequent distal metastasis with odd's ratio (OR) of 2.28, 2.34,

7.42, 10.41, 1.99, 3.33, 5.72, 6.05, 6.13 and 16.6 respectively. No such association were found with local recurrence.

Table 12: Mortality and Recurrences Based on Patient Demographics and Histological Patterns

Odd's ratio, CI and P-value									
	Death from disease			Distant metastasis			Local Recurrence		
Male vs Female	2.25	1.37-3.67	P<0.01	2.28	1.38-3.75	P=0.01	1.78	0.64-4.99	P=0.26
Age Group	N/A	N/A	P=0.34	N/A	N/A	P=0.17	N/A	N/A	P=0.23
Rectum vs colon	2.34	1.33-4.13	P<0.01	1.88	1.04-3.37	P=0.02	2.56	0.86-7.59	P=0.08
Right vs Left	0.9	0.52-1.55	P=0.71	0.86	0.5-1.49	P=0.69	0.64	0.19-2.13	P=0.46
Poorly differentiated	1.99	1.17-3.38	P=0.01	1.93	1.13-3.29	P=0.01	0.87	0.24-3.13	P=0.83
Mucinous	0.98	0.44-1.98	P=0.85	0.82	0.37-1.81	P=0.65	1.83	0.50-6.63	P=0.35
Signet cell	1.61	0.16-15.6	P=0.68	1.65	0.17-16.1	P=0.66			P=0.71
LVI	3.33	2.03-5.43	P<0.01	3.34	2.04-5.47	P<0.01	1.13	0.4-3.17	P=0.80
PNI	5.72	3.21-10.2	P<0.01	7.12	3.98-12.8	P<0.01	0.98	0.22-4.41	P=0.98
EVI	6.05	3.47-10.5	P<0.01	6.33	3.63-11.1	P<0.01	1.36	0.38-4.91	P=0.62
IED	6.13	2.95-12.7	P<0.01	4.81	2.31-10.0	P<0.01	1.96	0.43-9.18	P=0.37
N+ve	7.42	4.26-12.9	P<0.01	4.86	2.89-8.18	P<0.01	2.68	0.96-7.5	P=0.05
Stage IV	10.41	4.73-22.9	P<0.001	2.67	1.2-5.94	P<0.01	1	0.13-7.83	P=0.99
LR	16.6	5.21-52.9	P<0.001	5.36	1.95-14.7	P<0.01			
Distant Metastases	98.87	47.1-207.6	P<0.01				5.36	1.95-14.73	P<0.01
MSI	0.21	0.03-1.57	P=0.09	0.21	0.09-1.61	0.1	0.34	0.18-2.64	P=0.36

N+ve – Node positive disease; LVI – Lymphovascular involvement; PNI – Perineural invasion; EVI – Extramural venous involvement; IED – Isolated extramural deposit; LR – Local recurrance; MSI – Microsatellite instability.

2.5.2 Analysis of correlation

Univariate and multivariate analysis were used to predict survival or recurrence based on clinical features and histological findings. When univariate analysis

was performed, most clinical variable and histological variable seem to be a risk factor for cancer specific mortality (Table 13). However, when multivariate analysis was performed, only patients who were node positive, with metastatic disease at time of surgery, local recurrence or subsequent distal metastasis had a significantly higher risk of dying from the disease, with a OR of 4.28, 7.69, 16.91 and 28.72 respectively (Table 13). As for the risk of developing subsequent metastasis, the multivariate analysis showed that male patients, rectal tumours, nodal involvement, local recurrence, and perineural involvement were variables that were predictive of developing subsequent metastasis (OR of 1.98, 2.29, 2.11, 4.41 and 4.4 respectively). patients who developed subsequent metastasis was the only risk factor identified for developing local recurrence with an OR of 5.43 (Table 15).

Table 13: Univariate and Multivariate analysis for Cancer Specific mortality

Survival	Univariate Analysis			Multivariate analysis		
	OR	95% CI	P Value	OR	95% CI	P Value
Gender (Male)	2.25	1.37-3.64	<0.01	1.29	0.49-3.38	0.61
Age Group			0.34			0.44
Site (Rectum)	2.34	1.33-4.13	<0.01	1.87	0.57-6.17	0.31
Side (Right)	0.9	0.52-1.56	0.71	0.76	0.24-2.41	0.65
T staging			<0.01			0.67
N staging	7.4	4.26-12.91	<0.01	4.28	1.44-12.71	<0.01
M staging	10.4	4.74-22.92	<0.01	7.69	4.86-54.4	<0.01
Local Recurrence	16.61	5.21-52.94	<0.01	16.91	15.55-73.5	<0.01
Subsequent metastasis	98.71	47.08-207.61	<0.01	28.72	7.88-40.97	<0.01
Poorly differentiated	1.99	1.17-3.38	<0.01	2.0	0.43-6.35	0.27
Signet ring Tumour	1.6	0.17-15.59	0.69	0.25	0-209233	0.81
Mucinous Tumour	0.93	0.43-1.98	0.85	0.68	0.14-7.99	0.63
Lymphovascular Invasion	3.33	2.04-5.43	<0.01	0.85	0.30-3.81	0.78

Perineural Invasion	5.72	3.21-10.21	<0.01	1.30	0.41-6.12	0.68
Extra-mural venous invasion	6.05	3.47-10.54	<0.01	1.10	0.26-4.67	0.90
Isolated extra-mural deposit	6.13	2.95-12.74	<0.01	1.09	0.41-6.12	0.90

Table 14: Univariate and Multivariate Analysis for Subsequent Metastasis

Metastases	Univariate Analysis			Multivariate analysis		
	OR	95% CI	P Value	OR	95% CI	P Value
Gender (Male)	2.28	1.38-3.75	<0.01	1.98	1.09-3.55	0.02
Age Group			0.17			0.12
Site (Rectum)	1.88	1.04-3.37	0.03	2.29	1.09-4.86	0.03
Side (Right)	0.86	0.50-1.49	0.59	1.00	0.51-2.00	0.98
T Staging			<0.01			0.73
N staging	4.86	2.89-8.18	<0.01	2.11	1.10-4.03	0.03
M staging	2.67	1.20-5.94	0.02	1.28	0.41-4.03	0.76
Local Recurrence	5.36	1.95-14.73	<0.01	4.41	1.33-14.58	0.01
Poorly differentiated	1.93	1.13-3.29	0.02	1.70	0.67-3.23	0.14
Signet ring Tumour	1.65	0.17-16.07	0.67	2.17	0.12-27.55	0.60
Mucinous Tumour	0.82	0.37-1.81	0.62	0.79	0.27-2.35	0.79
Lymphovascular Invasion	3.34	2.04-5.47	<0.01	1.11	0.49-2.46	0.50
Perineural Invasion	7.12	3.98-12.75	<0.01	4.40	2.17-8.92	<0.01
Extra-mural venous invasion	6.33	3.62-11.07	<0.01	2.15	0.95-6.81	0.06
Isolated extra-mural deposit	4.80	2.31-10.02	<0.01	2.36	0.81-6.83	0.59

Table 15: Univariate and Multivariate Analysis for Local Recurrence

Local recurrance	Univariate Analysis			Multivariate analysis		
	OR	95% CI	P Value	OR	95% CI	P Value
Gender (M)	1.78	0.64-4.99	0.27	1.27	0.43-3.73	0.66
Age Group			0.27			
Site (rectum)	2.56	0.86-7.59	0.09	1.82	0.53-6.23	0.34
Side (Right)	0.64	0.19-2.13	0.47	0.54	0.14-2.10	0.37
T staging			0.51			

N staging	2.68	0.96-7.50	0.06	1.88	0.56-6.26	0.31
M staging	1.00	0.13-7.83	1.00	0.69	0.07-6.50	0.74
Subsequent metastasis	5.36	1.95-14.73	<0.01	4.63	1.71-17.21	0.01
Poorly differentiated	0.87	0.24-3.12	0.84	0.99	0.26-3.89	0.99
Signet ring	0.99	N/A	0.99	<0.01	N/A	0.99
Mucinous	1.83	0.50-6.63	0.36	2.35	0.59-9.26	0.22
LVI	1.13	0.40-3.17	0.81	0.99	0.26-3.80	0.99
PNI	0.98	0.22-4.41	0.98	0.42	0.07-2.38	0.32
Extra-mural venous invasion	1.36	0.38-4.91	0.64	0.68	0.12-3.93	0.67
Isolated extra-mural deposit	1.99	0.43-9.18	0.38	1.43	0.23-8.84	0.69

LVI – Lympho-vascular invasion. PNI – Perineural invasion.

2.5.3 Survival

The overall five-year survival within this cohort was 63.7%. Patients with colonic adenocarcinoma had a significantly higher five-year survival when compared to rectal adenocarcinoma of 66% versus 51.9% (Figure 9). The five-year overall survival according to T staging were 79% for T1, 74.3% for T2, 66.1% for T3 and 42.9% for T4. Whereas the five-year overall survival according to overall staging was 77%, 74.3%, 52.6% and 20% for stage I, II, III and IV respectively. These differences were statistically significant with a P-value of less than 0.05. When patients were stratified into three different age group (Group 1, <50; Group 2, 50-79; Group 3, >80), the five-year overall survival were significantly better for younger patients; 72.7%, 68.3%, 50.3 for group 1,2 and 3 respectively.

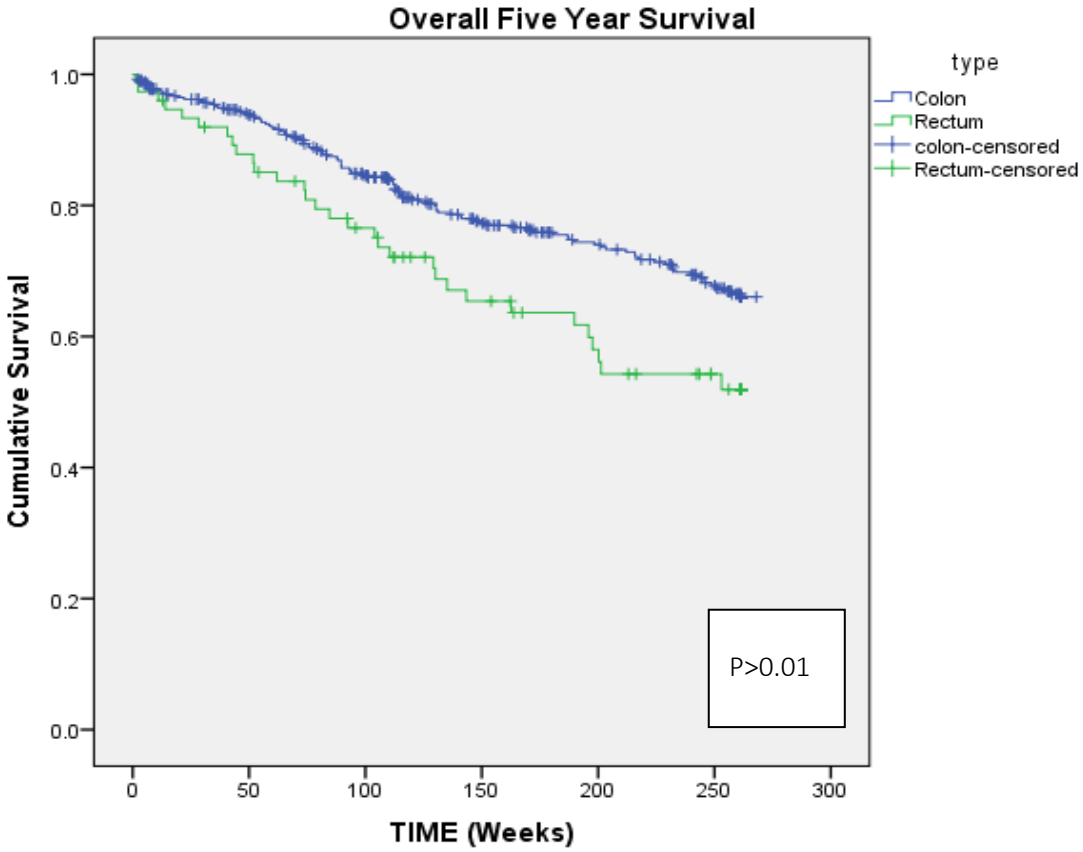

Figure 9: Kaplan-Meier Survival Analysis of Patients with Colon and Rectal Cancers

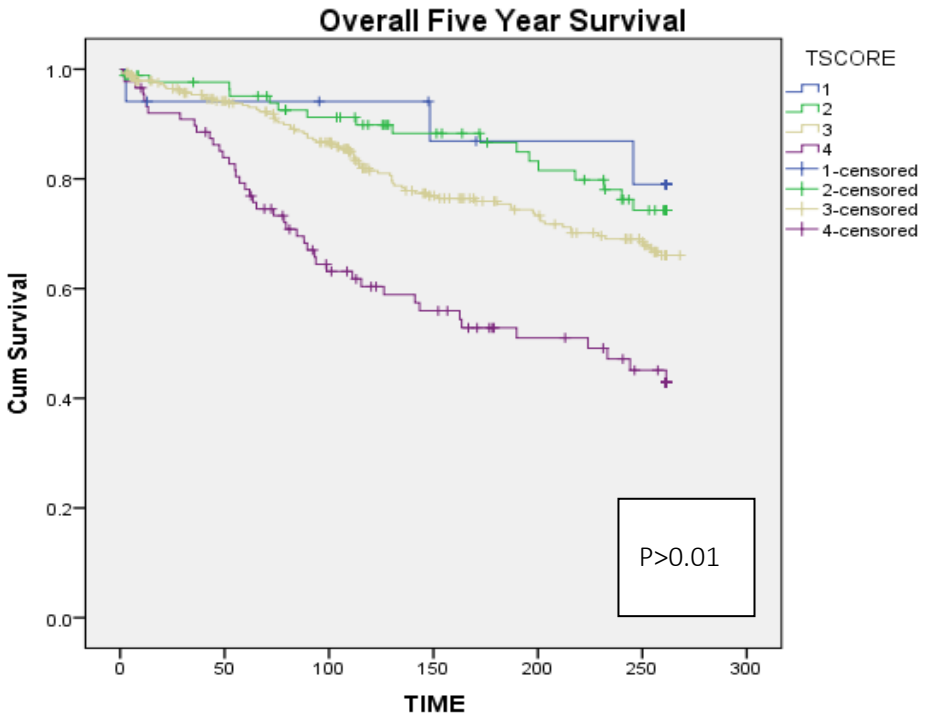

Figure 10. Kaplan-Meier survival analysis of patients with CRCs based on T staging

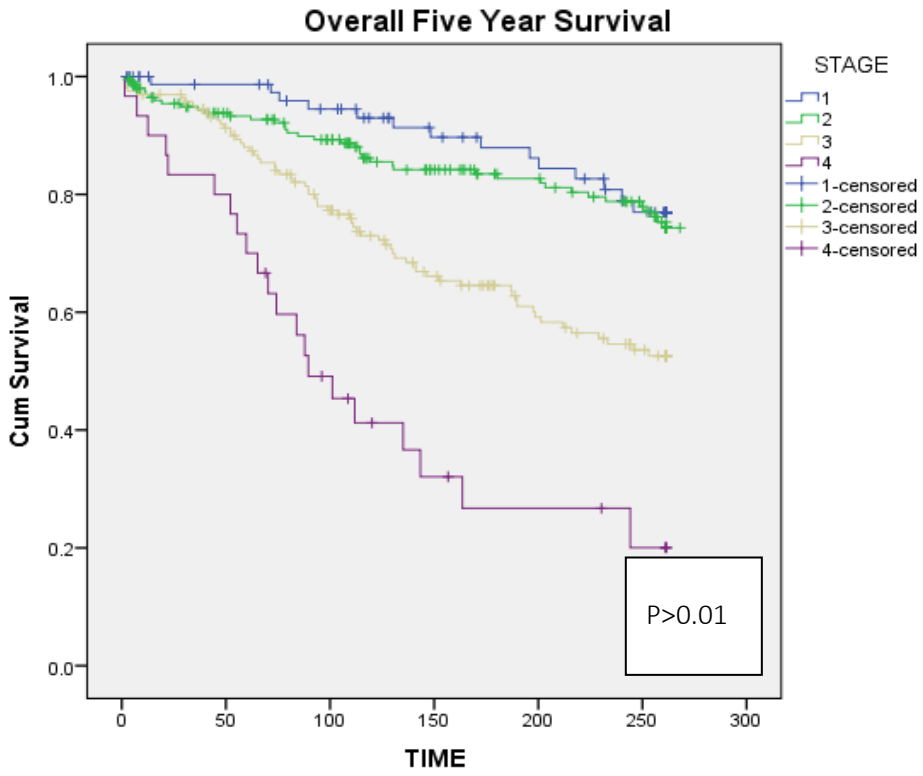

Figure 11. Kaplan-Meier survival analysis of patients with CRCs based on TNM staging

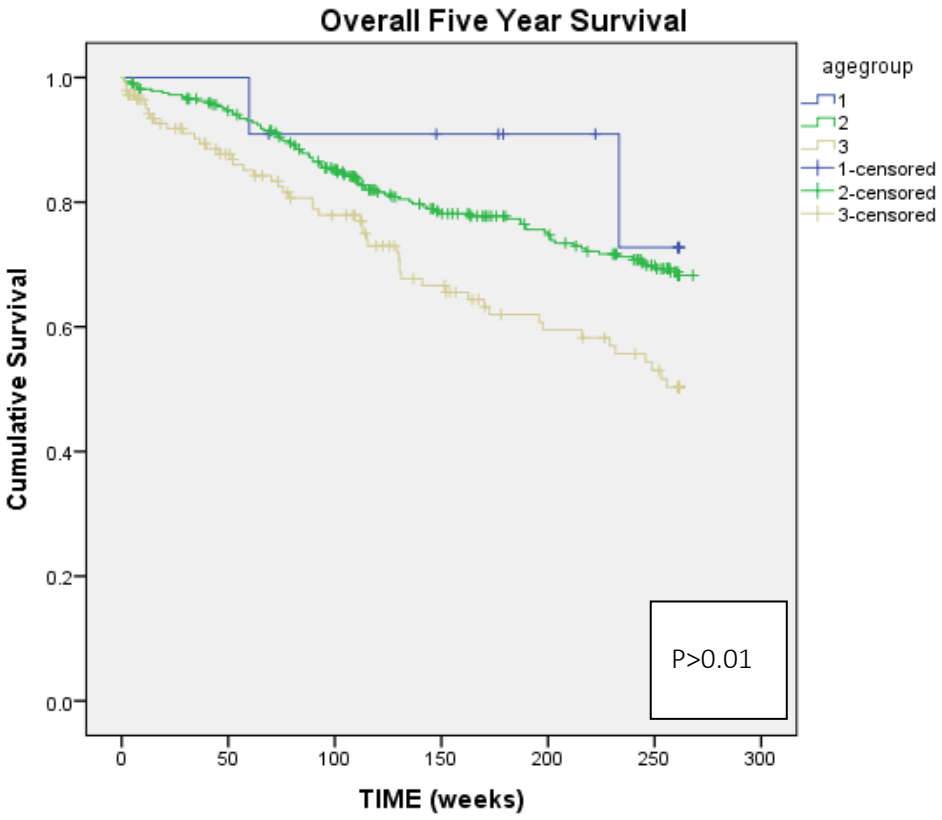

Figure 12. Kaplan-Meier survival analysis of patients with CRCs based on age group

2.6 Discussion

The demographics and survival outcomes of this study cohort are similar to what has been reported in the literature[15, 89]. CRC within this study was predominantly a disease of the older age group. However as with what is being observed in the literature, the incidence of CRC in patients who are less than 50 years is on the rise[90-94], and this trend is also reflected within this cohort, with 9 out of 11 patients who are less than 50 years of age, treated after 2008. CRC were predominantly right sided disease, and this is particularly true for female patients. Right sided tumours were more likely to be locally advanced and poorly differentiated. This is reflected in multiple studies[95-98]. Two meta-analyses [99, 100] have recently shown that right sided tumours have a different clinic-pathological presentation and have a significantly worse prognosis and higher risk of death when compared to left sided tumours. This adds to the ever-increasing clinical evidence that right sided tumours are of a separate entity [95, 98, 101, 102]. Studies have shown that right-sided tumours have different molecular-genetic makeup; lower expression of *c-myc*[103] and *TP53*[104] and a higher incidence of MSI[105]. Recent NGS and unsupervised clustering have shown that right-sided tumours are more commonly seen in some subtypes of CRCs[75, 78, 85]. However, the clinical outcome of these tumours was not hugely affected, with similar local recurrence and survival outcomes as shown by the multivariate analysis performed within this study. By merely classifying tumours to left and right-sided is an over-simplistic alternative and as a result, the prognosis and survival outcomes of reported epidemiological studies vary significantly[99].

Looking at survival, results are comparable to other Australasian and North American results[15, 89, 106, 107]. When compared with the SEER database, the five-year survival for local and regional disease were similar, however the five-

year survival for patients with metastatic disease within this study was higher (20% versus 16%). Survival was marginally better when different age groups were compared; 72.7% versus 65% for young patients.

More importantly this study showed that N staging, M staging and disease recurrence were the only significant risk factors for dying from CRC. This adds to the ever-increasing contradictory evidence within the available literature regarding which clinicopathological variables are predictive for survival. Ponz de leon et al [108] and Park et al [109] suggested that only morphological variables are the only important variables that are predictive of survival, however Nissan et al [110] suggested by their multivariate analysis that pathological variables such as LVI, and tumour grading are predictive of survival outcome. This contradiction highlights the difficulty of predicting patient outcome base on the traditional clinic-pathological classification system. The difference in cohort demographic, geographical and environmental variation and underlying heterogeneity in genetics accounts for this vast difference in outcome. Hence there needs to be a more robust, universal classification which accounts the molecular genetic differences in CRC, to better predict and prognosticate CRCs.

There are inherent biases to this study. This study was part of a larger study to externally validate the CMS classification system, as such, certain exclusion criteria such as neoadjuvant therapy, hereditary tumours and synchronous tumours were not avoidable. Large proportions of stage IV CRCs and emergency resections were excluded from the cohort as these tumours were either not operated on or the resection occurred at a time not conducive for immediate freezing and storing of specimens. This together with the inclusion and exclusion criteria set at the beginning of the study meant that a substantial proportion and important segment of the population with CRCs including large proportions of

patients with rectal cancers have been excluded, ultimately effecting the prognosis and outcome of the study. Small numbers in both emergency resections and stage IV disease may artificially improve outcomes as reflected in the five-year survival rates shown within this study. The other major limitation of this study is the retrospective nature of this study. Though the tissue stored within CSTB were prospectively stored, the clinical data collected in this study was retrospectively collected. The accuracy of the collected data is dependant accuracy of the record keeping and pathological reporting, particularly between 2002 to 2006 where T staging and histological reporting was not standardized.

Despite this, this study does provide an adequate insight to the clinical behaviour of our CRC population; more importantly it does high light the short comings of traditional classification system and the limited ability of traditional histological and clinical variables in prognosticating and predicting the clinical behaviour of CRC.

2.7 Conclusion

In conclusion, the demographic and survival outcome shown is comparable to results from other developed countries. The TNM system is good at prognosticating survival. Additional methods such as molecular classification may help further in prognostication.

3. External Validation of Consensus Molecular Subtypes of CRC - Demography and Histopathological Features Associated with CMS Subtypes

3.1 Abstract

Aim: To externally validate the Consensus Molecular Subtyping (CMS) of colorectal cancer (CRC) and to identify clinical and histological associations within each subtype.

Methods: 306 patients were selected from the 480 patients (discussed in chapter 2). Frozen tissues divided, and RNA extracted. Sample preparation, including library creation and ribosomal RNA depletion was carried out using Illumina TruSeq V2 reagents (NZGL, Massey University, Palmerston North). RNA sequencing carried out using Illumina HiSeq 2500 V4 platform. Raw sequence reads were checked and mapped to human reference genome. Gene expression profiles from each patient were used as input data to the publicly available CRC subtype classifier[85]. The clinicopathological, treatment outcome and 5-year follow-up data were collected retrospectively from patient notes.

Results: Of the 306 patients, 19.3% were CMS1, 45.4% were CMS2, 13.1% were CMS3 and 5.2% were CMS4. 17% of CRCs were not classifiable. CMS1 tumours were mainly right-sided, node-negative, poorly-differentiated and Microsatellite instable (MSI) tumours with a high proportion of mucinous histology. CMS2 tumours were predominantly left-sided tumours found in male patients and were mainly Microsatellite stable (MSS). CMS4 tumours were mainly found in younger patients with left sided tumours and present at an advanced stage. This

compares well to what was published by the Colorectal Cancer Subtyping Consortium (CRCSC)[85].

Conclusion: This study showed that the CMS classification is reproducible on a large scale and showed robust and distinct clinical and histological features within each subtype. More effort is required to investigate the relative absence of CMS4.

3.2 Background

The cost associated with next generation sequencing (NGS) have decreased significantly and with it an increased uptake in genome wide analysis of CRCs. This has led to a flurry of new molecular classification of CRCs[67, 73-80, 85]. The most promising of which is the CMS classification published by the CRC Subtyping consortium (CRCSC)[85], who pooled data from 18 international databases. Four molecularly and genetically distinct subtypes with distinct phenotypes and clinical behaviour were described. If reproducible, it is hoped that it allows further risk stratification and prognostication of CRCs, enabling decisive clinical management, particularly in patients with stage II and III disease. Multiple studies have been conducted to evaluate the robustness and reproducibility of this classification [87, 111-116]. Building on the work performed by Purcell et al[87], we aim to externally validate the accuracy of the CMS classification, and in this chapter, focus on the clinical and histological features associated with each individual subtype.

3.3 Methods

3.3.1 Patient selection

Some 306 patients were selected from the previously described 480 patients (Chapter 2). Due to funding restrictions, the initial recruitment target of 500 patients was reduced to 300. As a result, recruitment was focused heavily on patients that were less than fifty years of age and greater than eighty years of age. All patients that were excluded from the 480-patient cohort were patients aged between fifty to seventy-nine years of age.

3.3.2 Nucleic acid extraction, sequencing and classification

Patients were consented for CRCs to be stored in the Cancer Society Tissue Bank (CSTB) prior to surgery. Tumour tissue was flash frozen with liquid nitrogen after resection and stored at a temperature of -80° Celsius. Selected tissue was then transferred to RNAlater ICE (Qiagen N.V. Germany) and stored at a temperature of -20° Celsius. Twenty milligrams of frozen tumour tissue was divided, RNA extracted using RNeasy® kit (Qiagen N.V. Germany). Quantification of purified nucleic acid performed using NanoDrop 2000c spectrophotometer (Thermo Scientific, Asheville, NC, USA). Sample preparation, including library creation and ribosomal RNA depletion was carried out by using Illumina TruSeq V2 reagents. RNA sequencing carried out using Illumina HiSeq 2500 V4 platform. Raw sequence reads were quality controlled and mapped to human reference genome. Gene expression was quantified based on the number of reads mapped to particular gene loci. Gene expression profiles from each patient were used as input data to the publicly available CRC subtype classifier (v1.0.0, https://www.synapse.org/#!Synapse:syn4961785)[85] and classified into 4 subtypes.

3.3.3 Clinical data collection

The clinicopathological, treatment outcome and five-year follow-up data were collected retrospectively from patient notes. Tumours located proximal to the splenic flexure were documented as right sided tumour, tumours located distal to the splenic flexure and proximal to the rectosigmoid junction were documented as left sided tumour and tumours located distal and including rectosigmoid junction were documented as rectal tumours. In terms of histological findings, tumours that contained more than 50% mucin were

documented as mucinous tumours while tumours containing more than 50% signet cells were documented as signet cell tumour.

3.3.4 Data analysis

Data was entered into and entered into a custom-built Microsoft Access (Microsoft, Redmond, Washington, USA) database. Statistical analysis using Chi-square was carried out for categorical data and Kruskal-Wallis test used for continuous data. These were performed using SPSS® version 20 (IBM Corp®). A P-Value of <0.05 was deemed significant.

3.3.5 Ethics approval

The study protocol was approved by the University of Otago Human Ethics Committee (Ethics Approval Number H16/037).

3.4 Results

Of the 306 patients included in the analysis, 47.1% (144) were male and 52.9% (162) were female. The median age was 73.9 (range 29-92) years of age. 3.9% (12) of patients were aged 50 or less and 28.8% were aged 80 or more. 96.4% (295) were Caucasian, 2.9% (9) were Maori and 0.7% (2) were of Asian descent. In terms of the distribution of the tumours, 44.1% (135) were right sided, 37.9% (116) were left sided and 18% (55) were rectal tumours. 17.3% (53) of patients had stage I disease, 41.2% (126) had stage II disease, 34.3% (105) had Stage III disease and 7.2% (22) had stage IV disease. Histologically 17.3% (53) were poorly-differentiated and 10.5% (32) were mucinous. Table 16 shows the demographics and histology of our cohort in detail.

Table 16: Demographics and Characteristics of patients

			n	Percentage
Demography	Gender	Male	144	47.1
		Female	162	52.9
	Age	Median	73.9	
		≤50	12	3.9
		51-79	206	67.3
		80	88	28.8
	Ethnicity	European	295	96.4
		Maori	9	2.9
		Asian	2	0.7
Staging and Histology	Site	Right	135	44.1
		Left	116	37.9
		Rectum	55	18
	Stage	I	53	17.3
		II	126	41.2
		III	105	34.3
		IV	22	7.2
	Poorly differentiated		53	17.3
	Mucinous		32	10.5
	Signet cell		2	0.7
	Lymphovascular invasion		100	32.7
	Extramural venous invasion		45	14.7
	Isolated extramural deposit		24	7.8
	Perineural invasion		37	12.1

19.3% (59) patients were classified as CMS1, 45.4% (139) as CMS2, 13.1% (40) as CMS3 and 5.2% (16) as CMS4. 17% (52) of all patients were not classifiable. Table 17 represents the distribution of patients according to the four subtypes and how it compares to the CRCSC[85].

Table 17: Proportion of Consensus Subtypes within our study and CRCSC

	Total	CMS1	CMS2	CMS3	CMS4	Unclassifiable
Our Study	306	19.3%	45.4%	13.1%	5.2%	17.0%
Guinney et al		14%	37%	13%	23%	13%

Table 18: Demographics and Characteristics of patients that were classifiable in this study and from the CRCSC

		Our Cohort		Consensus Consortium		P-Value
		n=254	**Percentage**	**n**	**Percentage**	
Gender	Male	118	46.4	1536	54	0.02
	Female	136	53.5	1308	46	0.02
Age	Median	73.9		66		
Site	Right	111	43.7	1034	39	0.16
	Left	95	37.4	1219	46	0.06
	Rectum	48	18.9	398	15	0.10
Stage	I	48	18.9	354	12	<0.01
	II	109	42.9	1151	39	0.22
	III	81	31.8	1210	41	<0.01
	IV	16	6.3	236	8	0.39
Poorly differentiated		49	19.3	88	16	0.27

CRCSC data obtained from Guinney et al[85], Supplementary table 5.

As shown on Table 18, other than having proportionately more females, more stage I disease and less stage III disease, the demography, clinical and pathological characteristics of this cohort is largely similar to that of the CRCSC. By comparing the clinicopathological feature between each consensus subtypes (see Appendix Table 31), CMS1 tumours were significantly associated with female gender (OR 2.95, P <0.01), right-sided tumours (OR 2.42, P<0.01), node-negative disease (OR 1.75 P=0.01), poorly-differentiated (OR 4.78, P<0.01), mucinous tumour (OR 3.57, P<0.01) and a higher proportion of MSI tumours (OR 9.9, P<0.01). These patients were also less likely to receive adjuvant treatment (OR 1.76, P=0.03). CMS2 tumours were significantly associated with male patients (OR1.61, P<0.01), left-sided tumour (OR 1.92, P<0.01) and MSS tumours (OR 1.15, P<0.01). There were no significant clinicopathological characteristics associated with CMS3 other than the fact that these were associated with larger tumours and higher T-staging (P<0.01). CMS4 was associated with left-sided tumours (OR 2.42, P=0.05) and advanced overall staging (P=0.03). Aside from CMS4 tumours

having a higher proportion of isolated extramural deposits, there were no significant difference in any other histological features between the four subtypes. There was no significant difference in the local recurrence rates and subsequent distant metastasis between the four subtypes. Table 19 summarises the clinicopathological features of each consensus subtypes found in our study and that of the CRCSC.

Table 19: Summary of clinicopathological findings associated with individual subtypes and comparing with the CRCSC[78]

CMS1	Older	Female	Right sided	Node Negative	Poorly Differentiated	Mucinous	MSI
CMS1	older	Female	Right sided		High grade tumour		MSI
CMS2	younger	Male	Left sided		Not Poorly Differentiated		MSS
CMS2		Male	Left sided		Lower grade tumour		
CMS3					Advanced T-staging		
CMS3			Right sided				
CMS4	Younger	Advance Overall staging	Left sided	Node positivity	Metastatic Disease on Diagnosis	Extramural Deposit	
CMS4	Younger	Advance Overall staging	Left sided		High grade tumour		MSS

White rows represent findings from our cohort and blue rows represent findings from the CRCSC.

3.5 Discussion

The key findings within this study were that CMS1 tumours occurred mainly in females and were mainly right-sided and poorly-differentiated; CMS2 tumours were mainly found in younger, male patients and were less likely to be poorly-differentiated; CMS4 were mainly left-sided tumours associated with advanced staged malignancies. This matches the findings reported by the CRCSC[85] and

by multiple other studies[87, 90-94, 111-116]. This study represents one of the larger studies to externally validate the Consensus Molecular Subtypes by utilizing RNA-derived sequencing data.

There were proportionately more tumours classified as CMS1 and 2 and only 5.2% of tumours classified as CMS4. There were also more tumours that were unclassifiable at 17% compared to 13% as published by the CRCSC[85]. The reason for a higher proportion of CMS1 and CMS2 tumours and comparatively less CMS4 tumours could partly be due to the recruitment process. Due to funding restrictions, there was only sufficient funds to process approximately 300 patients as oppose to the initial plan of 500 patients. As such recruitment was focused mainly on patients that were 50 years or less and 80 years or more. The rationale for this came from recent publications which cited the increasing incidence of CRCs in the very young and old patients[117, 118]. With higher proportions of younger and older patients, it is not surprising that there was a higher proportion of CMS1 and CMS2 tumour as these tumours are associated with older and younger patients respectively. The other possible explanation could be due to the slight differences in methodology the cohort. As explained by Purcell et al[87], the CRCSC utilised TCGA data which has since been updated. They also utilised data from micro-array derived datasets which is different to the current study, which is strictly RNA-sequencing derived. Thus, potentially leading to a difference in the classification of the tumours.

With regards to the smaller numbers of CMS4 tumours in this study (5.2% as oppose to 23%), recent evidence suggest that the presence of EMT-associated genes seen in CMS4 tumours may reflect upregulated genes derived from fibroblast and mesenchymal cells present in the stromal background rather than directly from the tumour itself[55, 72, 87, 88]. This together with improvements in techniques involved in nucleic extraction and purification, may lead to less

tumours being classified as CMS4 tumour or the complete absence of it. This would clearly limit the clinical applicability of the CMS classification as a whole.

The second reason for the smaller number of CMS4 tumour is due to the fact that patients that had neoadjuvant therapy were excluded. Trumpi et al[112] concluded in their study that neoadjuvant therapy induces a mesenchymal phenotype in residue tumour cells and as such may lead to an increase in CMS4 subtypes. With exclusions of neoadjuvant treatment, one would thus expect a reduction in the number of CMS4 tumours. This effect will be explored in more detail in Chapter 6.

This study showed that the clinical and histo-pathological characteristics of patients with tumours that were classifiable into the four CMS subtypes were largely similar to that of the CRCSC[85]. The only difference was that in this study there was a higher female ratio, a higher rate of stage I disease and a lower rate of stage III disease. One could thus argue that with this slight difference in population, how could this study be used to externally validate the CMS classification. It is important to recognise that the population of both studies should be matched prior to classification and not after. The information on patients with non-classifiable tumours from the CRCSC is not publicly available for analysis. Thus, making it difficult to ensure that the clinical and histopathological characteristics of patients in both cohorts are comparable prior to classification.

Despite this, this study has successfully shown that individual CMS subtypes have distinct clinical features and reiterates the heterogenous nature of CRCs, re-enforcing the fact that traditional phenotype-based classifications are inaccurate and do not have the ability to prognostic and predict the behaviours of CRCs.

None of the CMS tumours had significant association with any histological features. MSI-H tumours are present in both CMS1 and CMS3 tumours.

3.6 Conclusion

In conclusion, by using RNA-derived sequencing data, successful validation of the Consensus Molecular Subtyping for CRCs was performed, and distinct clinical features associated with each subtype was reproduced. There is increasing evidence that disputes the presence of CMS4 tumours and more research into this field is required.

4. Prognosis and Survival Outcome Associated with CMS Subtypes

4.1 Abstract

Aim: To identify the prognosis and survival outcomes of the different Consensus Molecular Subtypes (CMS).

Methods: 306 patients were selected. Frozen tissue was divided, and RNA extracted. Sample preparation, including library creation and ribosomal RNA depletion was carried out using Illumina TruSeq V2 reagents (NZGL, Massey University, Palmerston North). RNA sequencing carried out using Illumina HiSeq 2500 V4 platform. Raw sequence reads were checked and mapped to a human reference genome. Gene expression profiles from each patient were used as input data to the publicly available CRC subtype classifier[85]. The follow-up data including five-year survival and recurrence were collected retrospectively from patient notes.

Results: The five-year survival rates for patients with CMS1, CMS2, CMS3 and CMS4 tumours were 67.6%, 68%, 55.5% and 41.1% respectively (P=0.03). Forty-five patients developed relapse. The local recurrence rates for CMS1, CMS2, CMS3 and CMS4 tumours were 3.9%, 3.6%, 7.5% and 0% respectively (P=0.55). The distant metastatic rates were 11.9%, 20.1%, 12.5% and 31.2% for CMS1, CMS2, CMS3 and CMS4 tumours respectively (P=0.47). The median survival after relapse (SAR) was 15.3 months, 15.1 months, 25.4 months and 4.72 months for patients with CMS1, CMS2, CMS3 and CMS4 tumours respectively (P=0.27).

Conclusion: The five-year overall survival rates were similar to those published by the Colorectal Subtyping Consortium (CRCSC)[85]. However, the TNM staging system is better than the CMS classification for predicting survival outcomes. This study was too small to make any meaningful assessments on the SAR, local recurrence rate and distal metastatic rates.

4.2 Background

The ability to accurately prognosticate and predict the clinical behaviour of colorectal cancers (CRCs) is the holy grail in the management of CRCs, particularly with regards to recurrence and survival. For many years clinicians have relied on clinico-pathological based classifications system, such as Dukes' and TNM, to help prognosticate and predict treatment outcome for patients suffering from CRCs. Multiple studies have shown the inadequacy and inaccuracies associated with such classifications[7-10]. The Consensus Molecular Subtyping for CRCs have been shown to be a robust and promising way of prognosticating CRCs[85]. In this chapter, we aim to replicate and validate the ability of CMS classification in prognosticating CRCs, particularly the survival and recurrence rates of each subtype.

4.3 Methods

The same 306 patients described in Chapter 3 were included. The process of nucleic acid extraction, sequencing and classification into the four subtypes has been described in detail in Chapter 3. Follow-up data, including survival, recurrence and distant metastasis were collected retrospectively from patient notes. Data was then entered into a custom-built Microsoft Access database (Microsoft, Redmond, Washington, USA). Statistical analysis using Kaplan-Meier survival was used to analyse five-year survival and recurrence rates. Cox regression analysis was used to assess potential independent prognostic factors associated with survival. These were performed using SPSS® version 20 (IBM Corp®). A P-Value of <0.05 was deemed significant.

4.4 Results

As described in Chapter 3, of the 306 patients included in the study, fifty-two tumours (17%) were unclassifiable. Of the remaining 254 tumours, fifty-nine (19.3%) were classified as CMS1, 139 (45.4%) were classified as CMS2, forty (13.1%) were classified as CMS3 and sixteen (5.2%) were classified as CMS4. The median follow-up period was fifty months (0.2 to 174 months). The five-year survival for patients with CMS1, CMS2, CMS3 and CMS4 tumours were 67.6%, 68%, 55.5% and 41.1% respectively (P=0.03). When the five-year survival of individual subtypes was compared against each other, only patients with CMS4 tumours had a significantly worse overall survival with a median survival of forty-five months (P<0.05). The five-year disease-free survival (DSF) was 67.6%, 64.9%, 59.9% and 37.5% for CMS1, CMS2, CMS3 and CMS4 tumours respectively (Figure 13).

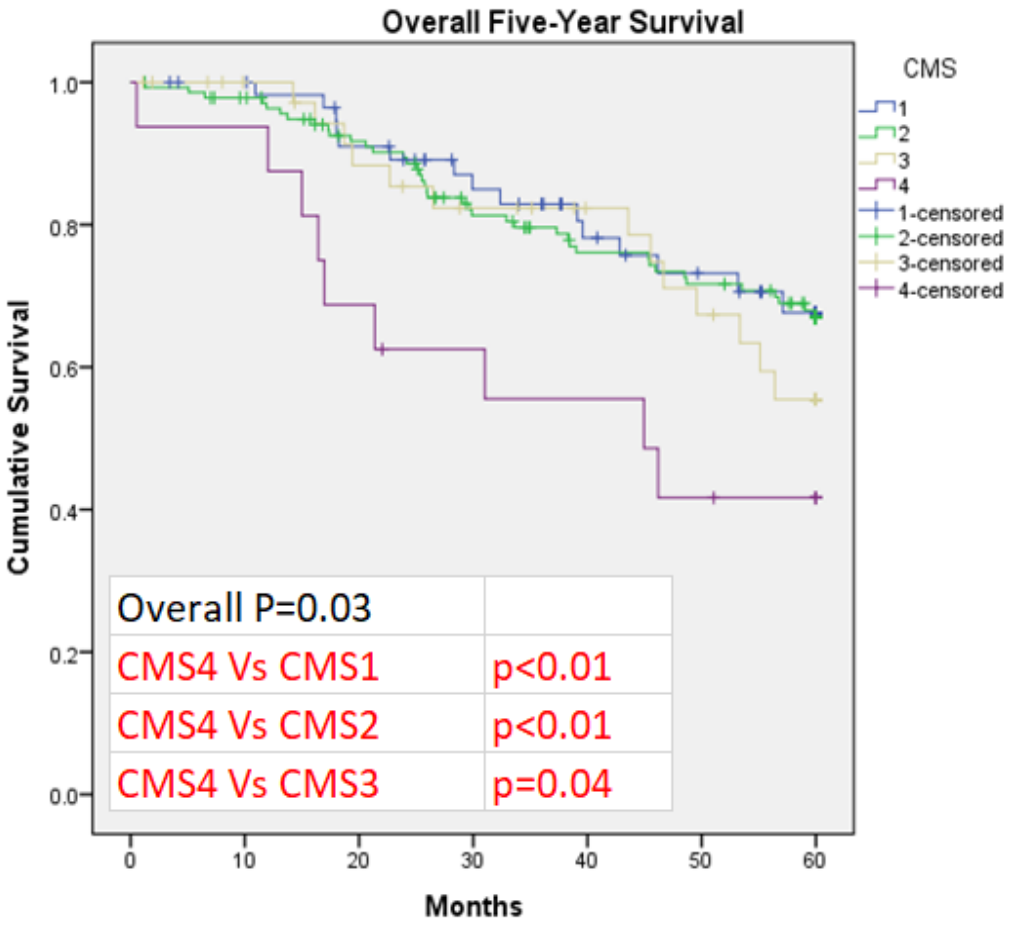

Figure 13: Overall five-year survival of the four Consensus Molecular Subtypes

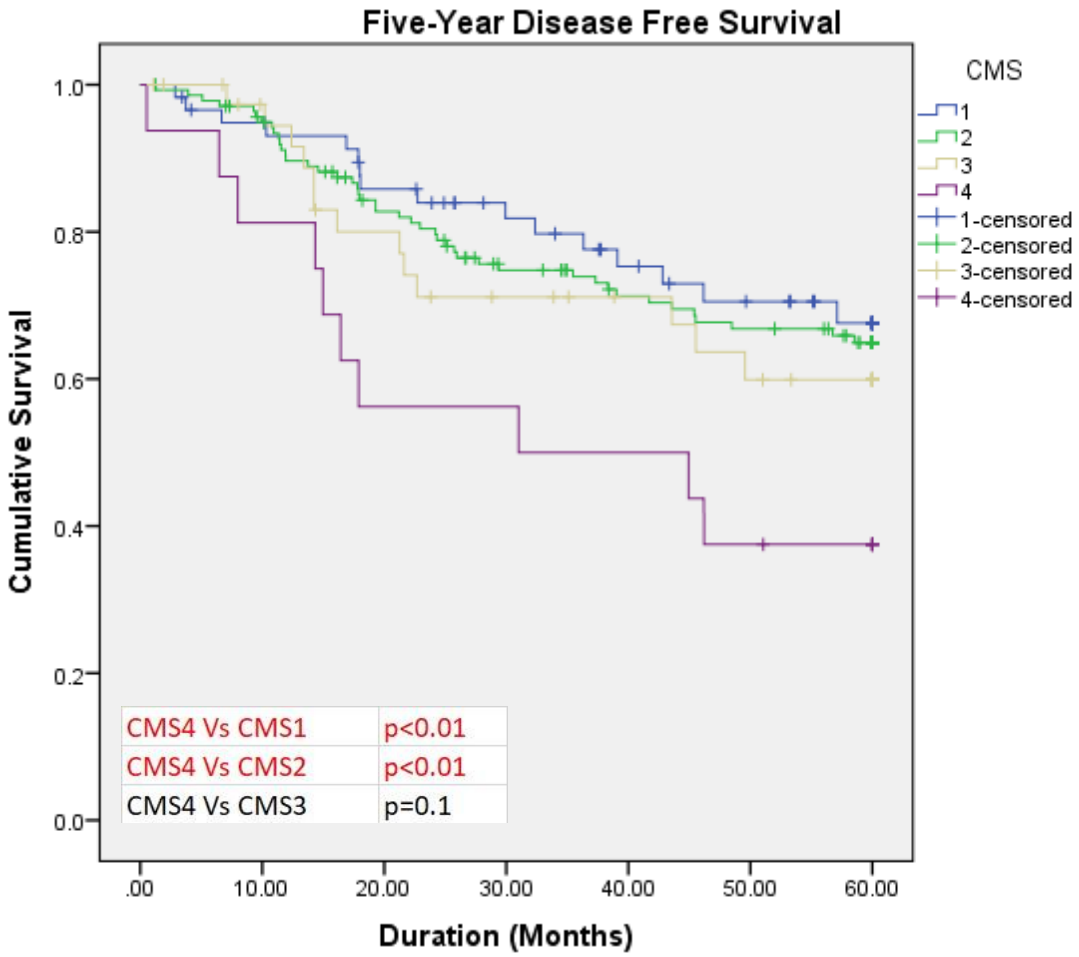

Figure 14: Five-Year Disease-Free Survival of The Four Consensus Molecular Subtypes.

Of the 306 patients, forty-five patients had relapse of their disease; nine had local recurrence and forty-two developed distant metastasis within the five-year follow up period. There was no significant difference in the median survival after relapse (SAR) which was 15.3 months, 15.1 months, 25.4 months and 4.72 months for CMS1, CMS2, CMS3 and CMS4 tumours respectively (P=0.27).

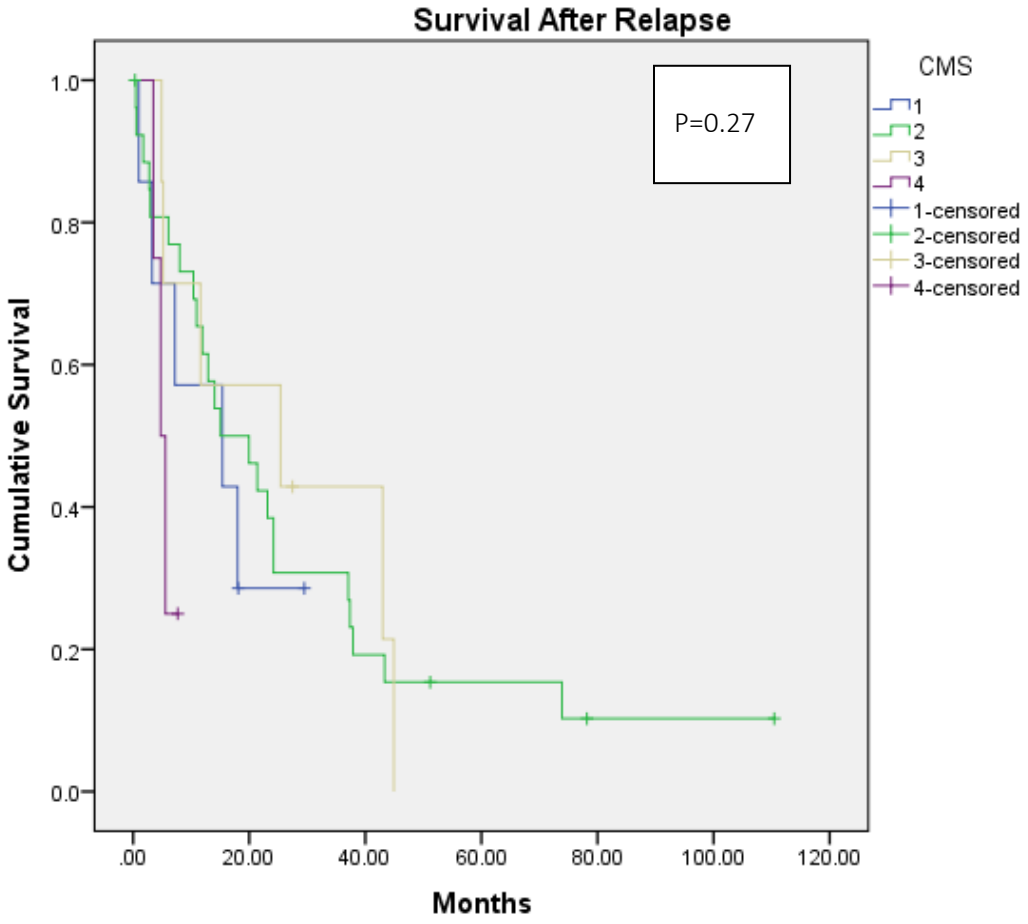

Figure 15: Five-year Survival After Relapse of each CMS Subtypes

Table 20: Univariate and multivariate Analysis for survival outcomes

Survival	Univariate Analysis			Multivariate analysis		
	OR	95% CI	P Value	OR	95% CI	P Value
Gender (Male)	1.93	1.12-3.33	0.18	1.15	0.55-2.31	0.71
Site (Rectum)	2.65	1.39-5.07	<0.01	1.37	0.62-3.03	0.44
CMS (CMS4)	2.55	1.32-6.50	0.04	1.94	0.59-6.35	0.27
T staging (T4)	3.73	1.95-7.31	<0.01	1.67	0.74-3.78	0.22
N staging	5.10	2.55-10.18	<0.01	2.37	1.02-5.48	0.04
M staging	6.92	3.37-14.23	<0.01	2.36	1.82-6.81	0.02
Local Recurrence	3.92	1.67-9.49	<0.01	1.18	0.39-3.51	0.77
Subsequent metastasis	18.62	9.08-38.16	<0.01	15.72	6.71-38.82	<0.01
Poorly differentiated	2.37	1.21-4.65	<0.01	1.94	0.75-5.17	0.16

Signet ring Tumour	1.43	0.32-1.53	0.97	1.25	0.28-1.97	0.81
Mucinous Tumour	0.44	0.11-1.82	0.25	0.72	0.14-5.43	0.46
Lymphovascular Invasion	2.54	1.37-4.71	<0.01	0.86	0.34-2.67	0.74
Perineural Invasion	3.86	1.93-7.72	<0.01	0.95	0.41-6.12	0.93
Extra-mural venous invasion	2.80	1.40-5.60	<0.01	0.44	0.12-1.64	0.22
Isolated extra-mural deposit	4.72	2.24-9.94	<0.01	2.78	0.94-8.23	0.06
MSI	0.48	0.01-15.94	0.46	0.87	0.02-10.96	0.67

Results in red signifies prognostic markers in both univariate and multivariate analysis.

Table 20 shows the results of the Cox regression analysis. In the multivariate analysis, only subsequent metastasis, N and M staging were identified as independent factors associated with poorer survival outcome. Subsequent metastasis, N and M staging had an Odds' ratio of 15.72, 2.37 and 2.36 respectively (P<0.05).

Table 21: The Local Recurrence and Metastasis in Different Subtypes During the Whole Study Period

	All samples	P	CMS1	CMS2	CMS3	CMS4
Local Recurrence	10 (3.9%)	0.55	2 (3.9%)	5 (3.6%)	3 (7.5%)	0 (0%)
Median time to recurrence (months)	21.8	0.77	53.5 (17-90.2)	24.7 (10.6-29.9)	21.6 (12.6-22)	-
Subsequent metastasis	45 (17.7)	0.20	7 (11.9)	28 (20.1)	5 (12.5%)	5 (31.2%)
Median time to subsequent metastasis (months)	16.6	0.15	10.5 (2.9-36.9)	18.9 (4.0-66.3)	13.6 (7.2-31.7)	14.6 (6.6-64.1)

There was no significant difference in the number of local recurrences or distant metastases between each of the CMS subtypes during the follow up period (Table

21). The local recurrence rate for CMS1, CMS2, CMS3, CMS4 tumours was 3.9%, 3.6%, 7.5% and 0% respectively (P=0.55). The median time to local recurrence was 53.5, 24.7 and 21.6 months respectively. The distant metastatic rate was 11.9%, 20.1%, 12.5% and 31.2% for CMS1, CMS2, CMS3 and CMS4 respectively (P=0.20) with a median time to metastasis of 10.5, 18.9, 13.6 and 14.6 months respectively.

4.5 Discussion

The overall survival shown in this study was similar to the CRCSC[85]. Patients with CMS4 tumours had significantly worse five-year overall survival of 41.1% (P=0.03). This was slightly lower compared to the five-year overall survival rate of 62% reported by the CRCSC[85]. A similar trend was observed in the five-year DFS when compared to the results from the CRCSC. CMS1 by far had the highest disease-free survival (75% from the CRCSC versus 67.6% from this cohort). When SAR was looked at, no significant differences between the four subtypes were found. CMS4 had the worse outcome with a median of 4.72 months. CMS1, and CMS2 showed similar median SAR at roughly 15 months. This is dramatically different to what was reported by the CRCSC. They reported that CMS1 had by far the lowest SAR of nine months followed by CMS4 of twenty-four months[85]. This difference could be explained by the small number of patients who developed recurrence in the current cohort and the substantial number of censored patients, which was 18%. This represents the biggest limitation of this study. By recruiting a larger number of patients and particularly the ones with recurrence, it is expected the results will mirror that of the CRCSC. The same could be said about the rate of local recurrence and rate of distant metastasis. Only nine and forty-two patients either developed local recurrence or distant metastasis. Though not significant, patients with CMS4 tumours had by far the highest number of distant metastasis and patients with CMS1 tumours had the

lowest number of recurrence and distant metastasis. This could explain why CMS1 has such a good prognosis and CMS4 has such a poor one.

This study and multiple other studies[75-80, 85, 119] have shown that patients with CMS1 tumours have an excellent overall survival despite having poor histological features. This good prognosis could be due to the immune activation pathways associated with microsatellite instable (MSI) tumours[119]. The stroma of the tumour is highly immunogenic[85, 119, 120] and express prominent levels of lymphoid and myeloid-specific genes. It is this that leads to an increase in anti-tumour immune response associated with CMS1 tumour, thereby keeping the growth and spread of the tumour in check.

Despite having a favourable prognosis, CMS1 tumours have one of the poorest rates of survival after recurrence[85, 121]. The explanation for this is two-fold. Firstly, and from an immunological prospective, advanced MSI tumours develop a phenomenon known as the adaptive resistance[122]; These tumours obtain the ability to evade host immune responses. In a normal setting, regulatory T-cells function as immune modulators and suppress the inflammatory response to allow for self-tolerance[122]. These regulatory T-cells have been found within the stroma of MSI tumours and are at especially elevated levels in advanced MSI tumours [123-125]. This together with the upregulation of immune evasion mechanisms such as the inhibitory checkpoint molecule PD-1 ligand (PD-L1)[116], causes a down regulation of the anti-tumour response. Thus, allowing tumour growth, metastasis and subsequent poor prognosis. Secondly from a clinical prospective, metastatic MSI CRCs tend to present as a more advanced, unresectable state[126] and are less chemo-responsive particularly to 5-flourouracil (5-FU) based therapy[51, 127-129]. This together with evidence to suggest of subtype switching to CMS4 tumours in hepatic metastasis (discussed

in Chapter 6) may explain the lower resectability rates and subsequent poorer prognosis in patients with metastatic or recurrent CMS1 tumours.

CMS4 tumours on the other hand has the poorest overall survival, SAR and a high rate of subsequent metastasis. Overall five-year survival was only 41.1% and median SAR was only 4.72 months. This is due to the fact that CMS4 tumours are associated with enrichment of epithelial-mesenchymal transition (EMT) associated genes and have increased TGF-β activation and angiogenesis[85, 119]. The stroma of these tumours consists of a microenvironment rich in innate immune cells and fibroblasts[119, 130]. These carcinoma-associated fibroblasts produce a wide array of factors including proangiogenic factors and immune suppressive factors[116]. This suppresses the antitumoral effect provided by the innate T-lymphocytes. Furthermore, there is emerging evidence to suggest a subtype switching in hepatic metastasis[112] particularly switching from CMS2 tumours to CMS4 tumours, further reinforcing the aggressive nature of this subtype.

Multiple studies have shown the prognosticating ability of the CMS system[85, 88], however this study represents the first study comparing the prognosticating ability of the CMS classification against the traditional TNM system. The key difference in survival seen was mainly between CMS4 and the other CMS groups (Figure 13, 14). The prognosticating ability of CMS classification still does not outperform the traditional TNM staging system. As seen in the multivariate analysis (Table 20), only N, M status and subsequent metastasis were significant predictors for reduced survival outcomes. CMS was not a significant predictor for survival outcome. This limits the translation of this classification system into clinical practice. This could firstly be due to the small numbers of CMS4 tumours within this study. Having only 16 patients classified as having CMS4 tumours meant that the numbers were too low to show significant correlation with

mortality. However more importantly, as discussed in Chapter 3, is the validity of CMS4 tumour itself. Multiple studies have shown that the enrichment of the EMT-associated genes derives primarily from carcinoma-associated fibroblasts and its surrounding stroma[55, 72, 87, 88, 113] rather than tumour cells and as such puts the validity of CMS4 tumours into question. Li, Arnadottir and Dunne et al have all suggested that the location and number of tumour biopsies and the underlying intra-tumoural differences can undermine the accuracy of CMS[86, 113, 131]. With RNA extracted from a single 20mg sample of the tumour and without the ability to know whether the stroma of the tumour or the tumour itself is being sampled, the accuracy of CMS classification would be significantly affected. Multiple biopsies and analysis of the tumour itself might improve the accuracy of the classification. With the accuracy of the CMS classification and the validity of CMS4 tumours in doubt, it is hardly surprising that CMS4 tumour was not found to be an independent factor for survival.

4.6 Conclusion

In conclusion, this study has successfully shown distinct survival patterns of each individual CMS subtypes. However, the TNM staging system is better than the CMS classification for predicting survival outcomes. Larger numbers of patients with recurrent disease are required to validate the metastatic rates and the SAR for each subtype.

5. CMS subtypes and response to adjuvant therapy

5.1 Abstract

Aim: To evaluate the response and outcome of the different Consensus Molecular Subtypes (CMS) of colorectal carcinoma (CRC) to adjuvant therapy.

Methods: 306 patients were selected. Frozen tissue was divided, and RNA extracted. Sample preparation, including library creation and ribosomal RNA depletion was carried out using Illumina TruSeq V2 reagents (NZGL, Massey University, Palmerston North). RNA sequencing was carried out using the Illumina HiSeq 2500 V4 platform. Raw sequence reads were checked and mapped to human reference genome. Gene expression profiles from each patient used as input data to the publicly available CRC subtype classifier[85]. The 5-year follow-up data including survival and recurrence were collected retrospectively from patient notes. Computed Tomography (CT) Scans were retrospectively reviewed for tumour load and response to adjuvant therapy.

Results: Eighty out of 254 patients (26.1%) received adjuvant treatment, of which twenty-six (10.2%) received palliative therapy. Fourteen patients received palliative chemotherapy only and four patients received palliative radiotherapy only. There was no significant difference in the chemo-response rate between the four subtypes. CMS4 tumours had the poorest chemo-response with all patients being poor responders. CMS3 had the best response with 33.3% of its patients having a good response to chemotherapy. There was no significant difference in the DFS of patients who received adjuvant chemotherapy and those that who did not, however there was a trend towards a lower DFS in patients with stage II CMS1 tumours and who received adjuvant chemotherapy.

Conclusion: Given the relatively small study, no significant differences in the response to adjuvant therapy between the different Consensus Molecular Subtypes was noted. Patients with Stage II CMS1 tumour seem to have a reduced survival outcome when given adjuvant chemotherapy. TNM staging system remains the classification system of choice in terms of determining adjuvant treatment. Larger and well-structured studies are required to further evaluate this.

5.2 Background

CRCs are highly complex and heterogeneous disease. Deciding when to utilise adjuvant therapy and predicting response to adjuvant therapy has proven to be difficult. Traditionally, the decision to utilise adjuvant treatment has been based on TNM staging and high-risk histological features[6]. Other than microsatellite unstable (MSI) tumours showing distinctively poor chemo-responses to FOLFOX therapy (5-fluorouracil, leucovarin, oxaloplatin) [127-129], there are very limited tools to help clinicians predict the chemo-response of CRCs. Consensus Molecular Subtyping offers a promising tool to assist clinicians in the management of patients with stage II to IV disease. To better understand this, the aim of this chapter is to evaluate the response of individual CMS subtypes to adjuvant and palliative therapy.

5.3 Methods

This study was divided into two parts. The first part of the study was to compare the responsiveness of each CMS subtypes to palliative chemotherapy. The aim was to directly assess the response of individual CMS tumours towards chemotherapy and radiotherapy with disease in situ. The second part of the study was to assess the effectiveness of adjuvant treatment in the management of each CMS subtypes by comparing the rate of local recurrence, distant metastasis and survival outcomes post adjuvant treatment.

5.3.1 Patient selection

The same 306 patients described in Chapter 3 were included. From this cohort, patients who received adjuvant treatment, palliative chemotherapy or radiotherapy were identified and selected.

5.3.2 Nucleic acid extraction, sequencing and classification

The process of nucleic acid extraction, sequencing and classification into the four subtypes have been described in detail in Chapter 3.

5.3.3 Clinical data collection and analysis:

Five-year follow-up data of selected patients were collected retrospectively from patient notes. For the first part of the study, the size, location and distribution of the distant metastasis or local recurrence were measured on the CT scan at time of relapse. Post palliative treatment CT scans are routinely performed by oncologist to assess response to treatment. The size, distribution and location of the disease on CT scan was remeasured by the same person. The formal CT report was also taken into consideration. A reduction of tumour load by greater than 50% denotes good response, a stable appearance or a reduction of less than 50% denotes a poor response and progression of disease on CT scan while on palliative chemotherapy denotes non-responders.

For the second part of the study, data on local recurrence, subsequent metastasis and survival outcomes were collected for patients who underwent adjuvant chemotherapy or radiotherapy.

Data was then entered into a custom-built Microsoft Access database (Microsoft, Redmond, Washington, USA). Statistical analysis using Chi-Square analysis and Kaplan-Meier survival analysis to assess differences in treatment response between groups. These were performed using SPSS® version 20 (IBM Corp®). A P-Value of <0.05 was deemed significant.

5.4 Results

As described in Chapter 3, of the 306 patients included in the study, fifty-two tumours (17%) were unclassifiable. Of the remaining 254 patients, fifty-nine (19.3%) tumours were classified as CMS1, 139 (45.4%) were classified as CMS2, forty (13.1%) were classified as CMS3 and sixteen (5.2%) were classified as CMS4. During a median follow-up of fifty months (0.2 to 174 months), eighty patients (31.5%) received adjuvant treatment; seventy-five patients had adjuvant chemotherapy and seventeen patients had adjuvant radiotherapy. Of the eighty patients that received adjuvant treatment, twenty-six received palliative treatment. Fourteen patients had palliative chemotherapy only without radiotherapy and four received palliative radiotherapy only without chemotherapy. Eight patients received both. All adjuvant and palliative chemotherapy were 5-FU based therapy. Table 22 table 23 shows the demographics and treatment response in patients who receive palliative chemotherapy only and palliative radiotherapy only.

Table 22: Demographics, Histology and Chemo-response of Patients Undergoing Palliative Chemotherapy Only

	All samples		**CMS2**	**CMS3**	**CMS4**
	Statistics	**P**	**Statistics**	**Statistics**	**Statistics**
Demographics and Histology	n=14		n=9	n=3	n=2
Gender		0.90			
M	8 (57.1%)		6 (66.7%)	1 (33.3%)	1 (50.0%)
F	6 (42.9%)		3 (33.3%)	2 (66.7%)	1 (50.0%)
Side		0.40			
Right	4 (8.6%)		2 (22.2%)	1 (33.3%)	1 (50.0%)
Left	7 (50.0%)		5 (55.6%)	2 (66.7%)	0 (0.0%)
Rectum	3 (21%)		2 (22.2%)	0 (0.0%)	1 (50.0%)
Stage		0.08			
I	2 (14.3%)		0 (0.0%)	2 (66.7%)	0 (0.0%)
II	2 (14.3%)		1 (11.1%)	1 (33.3%)	0 (0.0%)
III	5 (35.7%)		4 (44.4%)	0 (0.0%)	1 (50.0%)
IV	5 (35.7%)		4 (44.4%)	0 (0.0%)	1 (50.0%)

Poorly Differentiated	3 (21.4%)	0.41	2 (22.2%)	0 (0.0%)	1 (50.0%)
Mucinous	1 (7.10%)	0.12	0 (0.0%)	1 (33.3%)	0 (0.0%)
Lymphovascular Involvement	7(50.0%)	0.51	5 (55.6%)	1 (33.3%)	1 (50.0%)
Extramural Venous invasion	6 (35.7%)	0.34	4 (44.4%)	0 (0.0%)	1 (50.0%)
Extramural deposit	2 (14.3%)	0.26	1 (11.1%)	0 (0.0%)	1 (50.0%)
perineural invasion	4 (28.6%)	0.41	3 (33.3%)	0 (0.0%)	1 (50.0%)
Local Recurrence	2 (14.3%)	0.52	2 (22.2%)	0 (0.0%)	0 (0.0%)
Subsequent Metastasis	11 (78.6%)	0.41	7 (77.8%)	3 (100%)	1 (50.0%)
Chemo-response		0.896			
Unknown	1 (7.1%)		1 (11.1%)	0 (0.0%)	0 (0.0%)
Good	3 (21.4%)		2 (22.2%)	1 (33.3%)	0 (0.0%)
Poor	9 (64.3%)		5 (55.6%)	2 (66.7%)	2 (100%)
Nil	1 (7.1%)		1 (11.1%)	0 (0.0%)	0 (0.0%)

Of the fourteen patients who underwent palliative chemotherapy only, five (35.7%) had stage IV disease at the time of initial surgery and eleven (78.6%) developed further distant metastasis. There were no CMS1 tumours within this group. There was no significant difference in the demographics, histological features and chemo-response between the three subgroups. Two out of the nine (22.2%) CMS2 tumours had a good response whereas six out of nine (66.7%) either had a poor response or no response at all. One out of the three (33.3%) CMS3 tumour had a good response and all two (100%) CMS4 tumour had a poor response to palliative chemotherapy.

Table 23: Responsiveness to palliative radiotherapy

n=4	**CMS1**	**CMS2**	**CMS3**	**CMS4**
Location of metastasis	Abdominal wall recurrance	Vertebral metastasis	Ileo-colic recurrence in	Vertebral metastasis

Response to radiotherapy	Good	Good	Poor	Poor

Of the four patients that solely received palliative radiotherapy only, two showed good response to radiotherapy (CMS1 and CMS2) and two showed poor response (CMS3 and CMS4).

Table 24: Comparing Rates of Distant Metastasis and Local Recurrence in Patients with Stage II CRCs and Who Received Adjuvant Chemotherapy

	n	Subsequent Metastasis	OR	P	Local Recurrance	OR	P
CMS1	2	1 (50.0%)	2	0.65	0 (0.0%)	0.89	0.62
CMS2	9	3 (33.3%)			1 (11.1%)		

OR – Odds' Ratio, P – P Value. No CMS3 or CMS4 was included in this table as they did no develop any relapse

Table 25:Comparing Rates of Distant Metastasis and Local Recurrence in Patients with Stage III CRCs and Who Received Adjuvant Chemotherapy

	n	Subsequent Metastasis	P	Local Recurrance	P
CMS1	9	2 (22.2%)	0.62	0 (0.0%)	0.64
CMS2	26	7 (26.9%)		2 (7.7%)	
CMS3	9	1 (11.1%)		0 (0.0%)	
CMS4	3	2 (66.7%)		0 (0.0%)	

OR – Odds' Ratio, P – P Value

Table 24 and Table 25 shows patients who have Stage II and III disease and who received adjuvant chemotherapy, there were no significant differences in the local recurrence rates or the rates of distant metastasis between the different Consensus Subtypes.

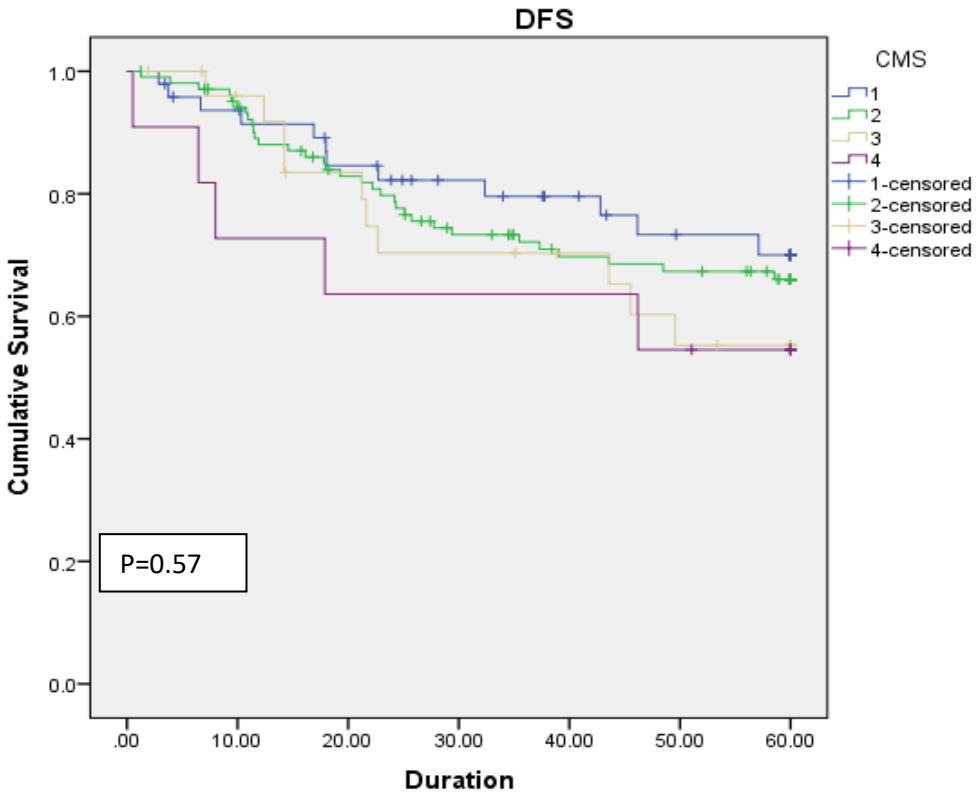

Figure 16: Five-Year Disease-Free Survival in Patients who received adjuvant Chemotherapy

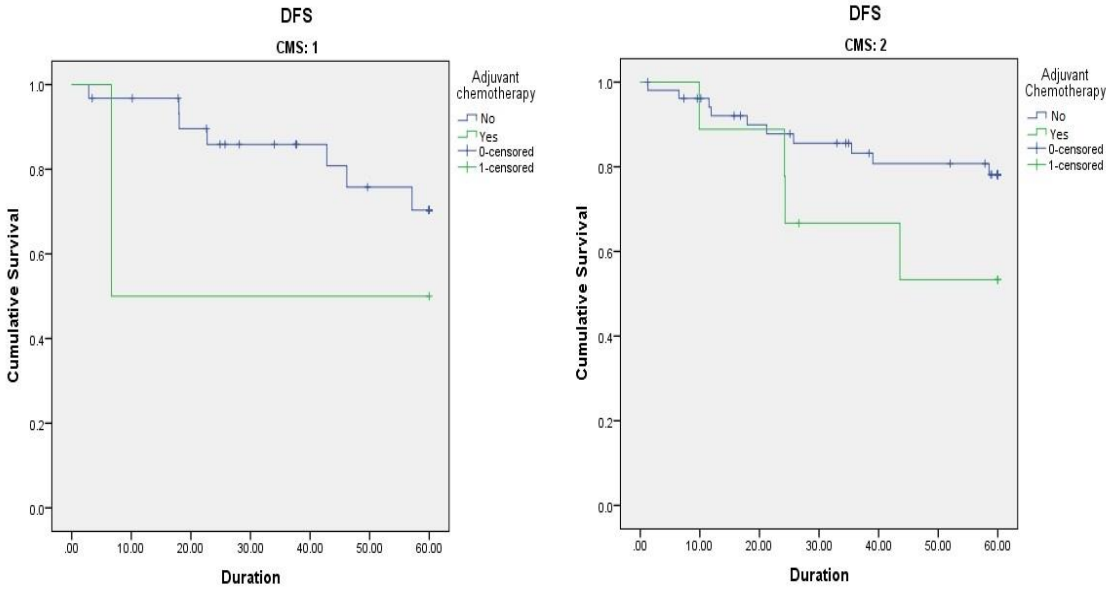

Figure 17:Comparing Disease Free Survival of Stage II CRCS Between Patients Who Received Adjuvant Chemotherapy and Those That Did Not

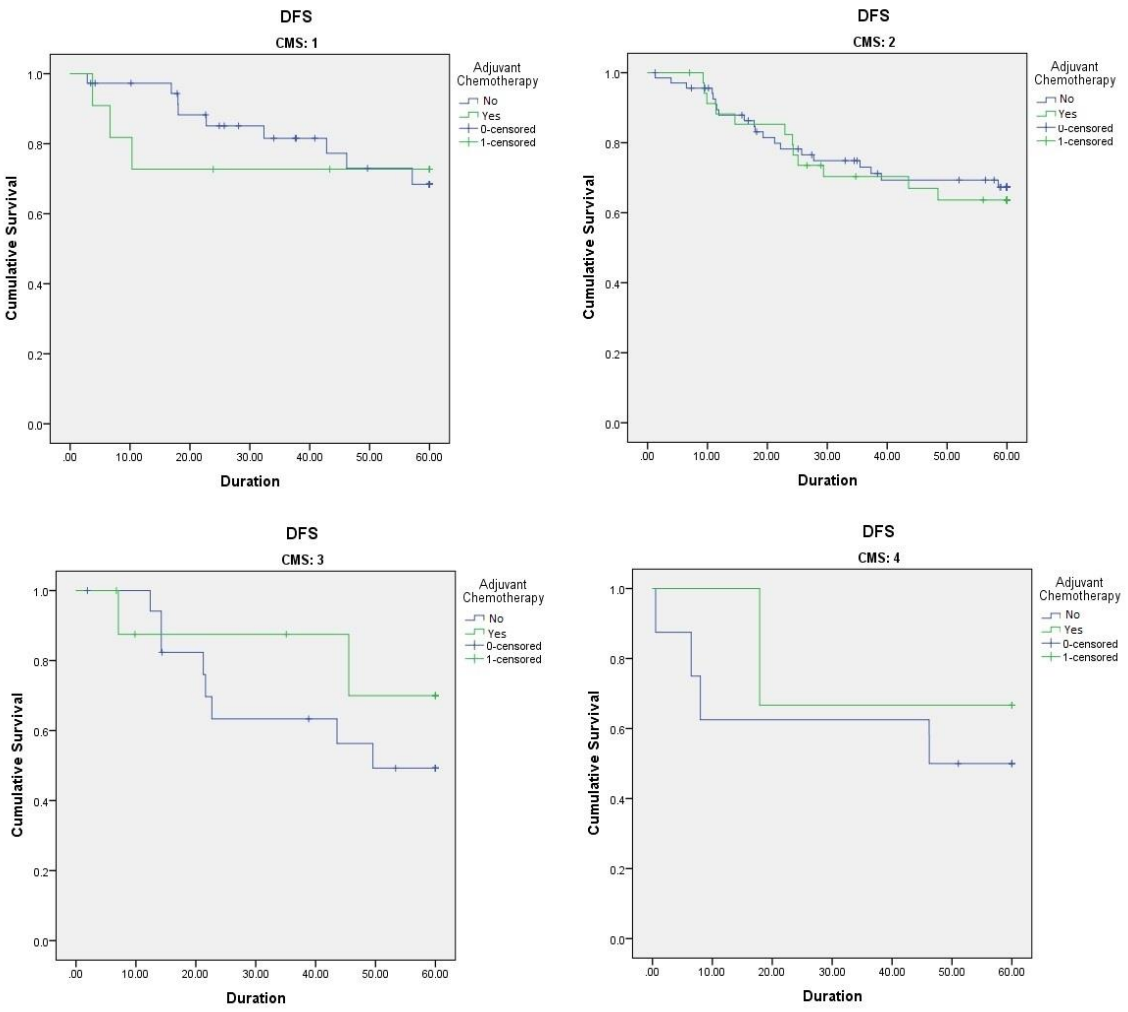

Figure 18: Comparing Disease Free Survival of Stage III CRCS in Patients Who Received Adjuvant Chemotherapy and Those That Did Not

When subset analysis on stage II and Stage III patients were performed, no significant difference was seen in the five-year DFS between patients who received adjuvant chemotherapy versus those who did not (Figure 17 and 18). There was a trend towards a lower DFS in patients with stage II disease and those who received adjuvant therapy (50% vs 70% for CMS1 and 53.3% VS 78.2% for CMS2). In Stage III disease, there was a trend towards a higher DFS for patients who received adjuvant therapy in all subtypes except CMS2 (72.7% vs 68.4% for CMS1, 63.6 vs 67.3% for CMS2, 70% vs 49.3% for CMS3 and 66.7% vs 50% for CMS4).

5.5 Discussion

To date, no studies have directly evaluated the response of different CMS subtypes to adjuvant treatment. This study attempted to do so in two parts. In the first part, the aim was to directly assess the response of individual CMS tumours towards chemotherapy and radiotherapy. To achieve this, only patients who underwent palliative treatment with disease in-situ were included. Analysis of patients who had single mode therapy (i.e. those that had only palliative chemotherapy or those that only had palliative radiotherapy) was performed to accurately distinguish response to chemotherapy or radiotherapy. As for the second part, the aim was to assess the response to adjuvant chemotherapy in each individual subtype by evaluating their local recurrence rate, rate of distant metastasis and DFS. Trying to achieve both parts of the study has led to the significant reduction in the number of patients included in the analysis, creating the biggest limitation to the current study (twenty-eight patients) and thus exposing this study to type II error and making it difficult to draw any meaningful conclusion.

It is interesting to see that CMS4 tumours had the poorest response with both the CMS4 patients responding poorly to palliative chemotherapy and radiotherapy. CMS3 and CMS2 tumours had similar mixed responses. This represents the first study to attempt to visualise directly the response in different CMS subtypes to palliative treatment. Recruiting larger numbers of patients who received palliative chemotherapy would have given a better picture of response, however the ability to accurately assess treatment response would still be challenging given the recent evidence suggestive of subtype changing that occurs in distant metastasis[112]. Trumpi et al suggested that there was at least 50% incongruency in the subtyping between primary tumour and the distant metastasis[112].

Unless all distant metastases are biopsied and subtyped in clinical practice, we would never be able to address this error accurately.

With regards to response to adjuvant chemotherapy, it is disappointing to see that there was no significant difference in the local recurrence rate, rate of distant metastases or five-year DFS amongst the CMS subtypes. In all CMS subtypes, a trend towards a higher DFS was observed in patients with stage III disease when compared to patients with stage II disease. This suggests that the TNM staging system remains a far superior tool in determining who receives 5-FU based adjuvant chemotherapy. Rodriguez-Salas et al and multiple other studies[88, 127-129] have shown that 5-FU based adjuvant therapy was only beneficial in Stage III disease, and in Stage II disease, 5-FU could have a negative effect on disease free survival.

Despite this, CMS subtyping may be useful in determining different antibody-based therapy. CMS1 tumours are highly immunogenic tumours with increased neo-peptide presentation to major histocompatibility complex 1 (MHC-1), therefore there is increasing evidence favouring the use of pembroluzimab (PD-1 inhibitor) in the treatment of CMS1 tumours, particularly those that are MSI[116, 119, 132, 133]. In 2015, a phase II trial showed a 40% response rate in MSI tumours to pembroluzimab[18].

CMS4 tumours on the other hand are highly enriched in epithelial-mesenchymal transition (EMT) associated genes[85, 119]. Multiple studies are currently underway to assess the efficacy of monoclonal antibodies against this, particularly the TGF-β signalling pathway (NCT02873195, NCT02291289, NCT02876224, NCT01633970)[120, 134]. Targeting this pathway will hopefully switch CMS4 tumours back to CMS1-like immune-responsiveness and thus allowing the introduction of checkpoint inhibitors such as pembroluzimab to

improve survival outcomes[88]. Anti-angiogenic drugs such as bevacizumab (anti-VEGFR antibody) have also been shown to be effective when used in combination with FOLFIRI (folinic acid, 5-FU, irinotecan) or FOLFOXIRI (folinic acid, 5-FU, oxaloplatin, irinotecan) in patients who have metastatic CRCs[88, 135, 136].

CMS2 and CMS3 tumours unfortunately are not immunogenic[85]. These tumours either show strong epithelial phenotype or have enrichments in metabolic pathways, especially *KRAS* is CMS3 tumours[85]. Increasing the immunogenicity of these tumours by promoting the expression of MHC-I through the usage of cobimetinib (a MAPK inhibitor) have been suggested in a few studies[120, 137]. This is particularly true of tumours enriched in *KRAS* mutation[137]. A small study of only twenty-two cancers showed that a combination of PD-1 inhibitor and cobimetinib resulted in partial response in 18% of patients[138].

Despite this, none of these studies were designed using CMS based classification. More large-scale studies are required to evaluate chemoresponsiveness of individual Consensus Molecular Subtypes to adjuvant therapy and antibody-based therapy.

5.6 Conclusion

In conclusion, this study has not shown significant difference in the response to adjuvant treatment among different CMS subtypes. TNM staging system remains the classification system of choice in terms of determining adjuvant treatment. The future may lie in targeted antibody treatments and large-scale studies are required to evaluate this.

6. CMS subtypes of primary tumour and subsequent liver metastases

6.1 Abstract

Aim: To evaluate the congruity in the Consensus Molecular Subtyping (CMS) of primary colorectal cancer (CRC) and hepatic metastasis.

Methods: 10 patients were selected. Frozen tissue of both primary and liver metastasis was divided, and RNA extracted. Sample preparation, including library creation and ribosomal RNA depletion was carried out using Illumina TruSeq V2 reagents (NZGL, Massey University, Palmerston North). RNA sequencing was carried out using Illumina HiSeq 2500 V4 platform. Raw sequence reads were checked and mapped to a human reference genome. Gene Expression profiles from each patient were used as input data to the publicly available CRC subtype classifier[85]. The 5-year follow-up data were collected retrospectively from patient notes.

Results: Nine out of ten patients had primary tumours that were classifiable. Seven were classified as CMS2, one was classified as CMS3 and one as CMS4. Four had incongruent classification in subsequent metastasis. Three out of four patients with incongruent classification had neoadjuvant chemotherapy prior to resection (P=0.02).

Conclusion: Despite having only ten patients, this study has successfully shown incongruency between the CMS Subtyping of primary CRCs and distant metastasis in a substantial proportion of patients. More importantly there was a

significant association with neoadjuvant therapy. Further investigation in this field is required.

6.2 Background

CRCs are complex disease with heterogeneous outcomes. Improvements have been made to the molecular classification and adjuvant therapy particularly targeted checkpoint inhibitors to improve outcomes. CMS1 and CMS4 tumours in particular show distinct prognosis and survival outcomes[85]. Effectiveness of treatment depends on the accuracy of classification of CRCs. It is still uncertain as to whether the molecular subtyping is preserved when metastasis occurs. A recent study has shown incongruency between primary tumour and subsequent metastasis[112]. In this chapter, the aim was to perform a pilot study to assess this potential and to lay the foundation for larger scale study to evaluate the congruency of CMS subtyping in both primary tumour and distant metastasis.

6.3 Methods

Due to funding restrictions, only ten patients were recruited into this study. The first ten patients who underwent primary colorectal resection and subsequent liver resection and who had both tumours stored in the Cancer Society Tissue Bank were identified and selected from the cohort of 306 patients described in Chapter 3. The process of nucleic acid extraction, sequencing and classification into the four subtypes of both primary tumour and liver metastasis were carried out in a similar fashion to what has been described in Chapter 3. Follow-up data, including survival, recurrence and distant metastasis were collected retrospectively from patient notes. Data was then entered into a custom-built Microsoft Access database (Microsoft, Redmond, Washington, USA). Statistical analysis using Fischer's exact test for categorical data. This was performed using SPSS® version 20 (IBM Corp®). A P-Value of <0.05 was deemed significant

6.4 Results

Ten patients were identified from the initial cohort of 306 patients. Of the ten patients, nine patients had primary CRCs that were classifiable, seven were CMS2, one was CMS3 and one was CMS4. There was no CMS1 tumour within this cohort. Table 26 shows the clinical characteristics of the nine classifiable patients. Four patients had synchronous liver metastasis and five had metachronous liver metastasis. Three patients underwent neoadjuvant chemotherapy prior to liver resection.

Table 26: Demography and clinical features of patients who had both colonic resection and liver resections

	Gender	Site	Type of metastasis	Neoadjuvant thrapy	Primary CMS	Liver CMS
1	Male	Right Colon	Metachronous liver metastasis	No	2	2
2	Male	Left Colon	Metachronous liver metastasis	Yes	2	4
3	Male	Left Colon	Metachronous liver metastasis	No	2	2
4	Male	Left Colon	Metachronous liver metastasis	No	3	Not classifiable
5	Female	Right Colon	Synchronous liver metastasis	Yes	2	4
6	Male	Left Colon	Metachronous liver metastasis	No	2	2
7	Female	Rectum	Synchronous liver metastasis	Yes	2	4
8	Male	Right Colon	Synchronous liver metastasis	No	4	2
9	Male	Right Colon	Synchronous liver metastasis	No	2	2

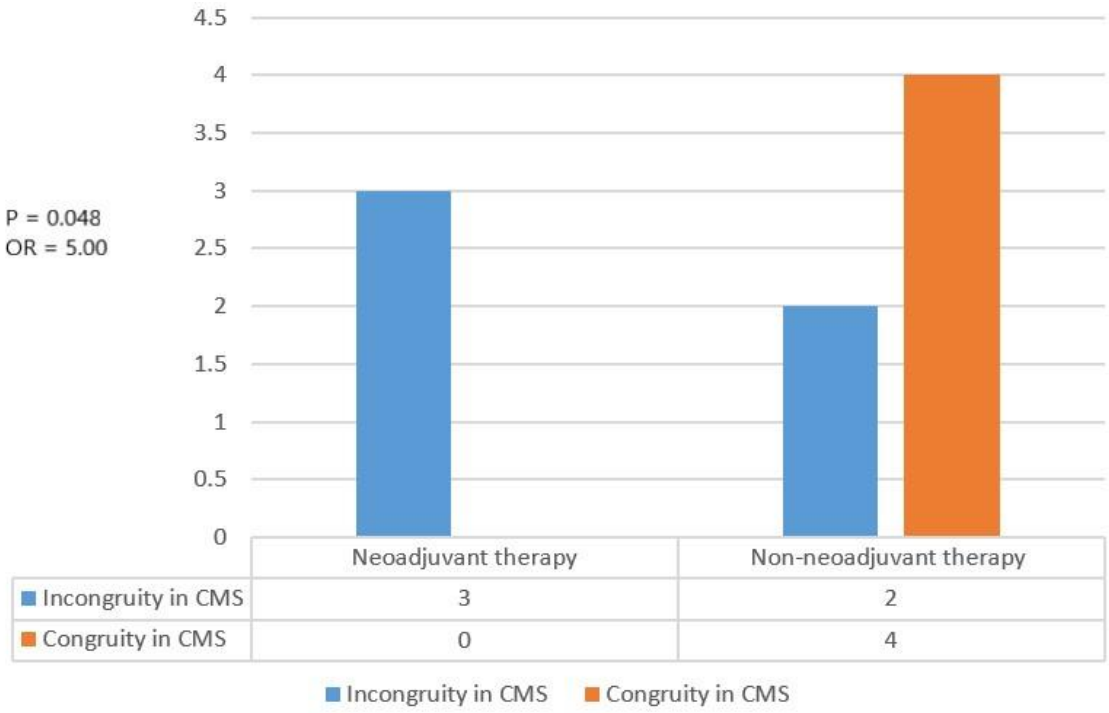

Figure 19: Congruency in CMS classification between primary CRC and liver metastasis

Of the nine patients who had classifiable primary tumour, incongruency occurred in five patients (Figure 19). As shown in Table 26, three "switched" from CMS2 to CMS4, one "switched" from CMS 3 to unclassifiable and one "switched" from CMS4 to CMS2. There was a significant association with neoadjuvant therapy. Three out of the five incongruent liver metastasis received neoadjuvant therapy prior to liver resection (P=0.048, OR=5.00).

6.5 Discussion

This study represents the second only study investigating the congruency of Consensus Molecular Subtyping between CRCs and hepatic metastasis. The other study published by Trumpi et al[112] utilised immunohistochemistry to classify tumours into epithelial-like tumours and mesenchymal-like tumour. This is the first study that utilise genome wide analysis to classify CRCs and hepatic metastasis into the four CMS Subtype.

The biggest flaw with this pilot study is that it only had nine patients, thus making it difficult to draw any meaningful conclusions. Despite this, incongruency in the molecular subtyping of hepatic metastasis was shown in a substantial proportion of the cohort (n=5). More importantly is the strong association with neoadjuvant chemotherapy. All three patients who received neoadjuvant therapy had incongruency in the molecular classification of liver metastasis, whereas four out of six patients who did not receive neoadjuvant therapy had congruent molecular classification in the liver metastasis. This finding is similar to that of Trumpi et al who suggested that neoadjuvant therapy leads to "switching" of subtyping within the liver metastasis[112]. This would support the idea of intra-tumoural difference that exists within a tumour (Chapter 1). Each tumour is made up of a multitude of sub-clonal cells, each having distinct biological and molecular properties with varying degrees of mutation[44]. It is hypothesized that selection pressure from neoadjuvant chemotherapy leads to a mesenchymal predominant sub-clonal population, hence a "switch" in CMS. This is also seen in other epithelial malignancies such as breast cancer[46, 139, 140]. Studies have shown that chemotherapy, particularly neoadjuvant therapy has led to an incongruency in the molecular classification between primary breast cancer and distant metastasis and a change in HER-2 status[139-141].

CMS4 tumours have been shown to have a worse survival and prognosis[85]. Whether this chemotherapy induced "switch" to CMS4 tumour will lead to a worse clinical outcome has yet to be assessed. The benefit resulting from the downstaging and improved resectability as a result from neoadjuvant chemotherapy must be weighed up against the potential poor prognosis associated with CMS4 subtype switching. Further large-scale studies are needed to first, validate the subtype incongruency associated with neoadjuvant

chemotherapy and secondly, to validate if this switch in subtype is associated with a poorer clinical outcome.

6.6 Conclusion

In conclusion, this pilot study showed potentially the presence of incongruences in the Concensus Molecular Subtyping between primary CRCs and hepatic metastasis. It raises the possibility that neoadjuvant chemotherapy induces switching in these metastases. This needs to be further assessed with large scale studies. How this will potentially impact on survival and prognosis needs to be further evaluated.

7. Conclusion Chapter

This study utilises RNA-sequencing-derived data extracted from CRCs stored within the Cancer Society Tissue Bank (CSTB) to externally validate the Consensus Molecular Subtyping (CMS) of colorectal cancer (CRC). We successfully externally validate The CMS of colorectal adenocarcinoma (CRC) and produced similar clinical-pathological results to Consensus Consortium. However, the prognostic and predictive value of this classification system remains in doubt.

The aim of Chapter 2 was to analyse the demographics of patients whose CRCs were stored within the CSTB and to assess if it was representative of the wider overall population. The epidemiology and survival outcomes of the current cohort are comparable to published results worldwide[15, 89, 106, 107]. Chapter 1 also reinforces the notion that no clinical or histological reliable variables other than advanced disease and recurrence were predictive of adverse outcomes. This highlights the need for a more robust and reproducible classification for prognosticating and predicting outcomes for CRCs.

The key findings in Chapter 3 are that firstly, the clinical and histological findings of each CMS subtype in this study matches that of the Colorectal Subtyping Consortium (CRCSC)[85]. Secondly, and more interestingly is that this study had a higher proportion of CMS1, CMS2, unclassifiable tumours and a lower proportion of CMS4. There is increasing evidence that disputes the presence of CMS4 tumours with suggestion that the presence of EMT-associated genes seen in CMS4 tumours may reflect upregulated genes derived from fibroblast and mesenchymal cells present in the stromal background rather than directly from

the tumour itself[55, 72, 87, 88]. This has direct implications on the prognostic accuracy of the CMS classification. More research into this field is required.

Chapter 4 successfully shown distinct survival patterns of each individual CMS subtype. However, the difference in survival was seen mainly between CMS4 and the other subtypes. More importantly, this study showed that the CMS classification system did not outperform the traditional TNM staging system in terms of prognosticating survival outcomes. This result limits the translation of this tool into current clinical practice.

In Chapter 5, due to the small numbers of the cohort, it is difficult to draw any meaningful conclusions from this study. No significant trend in response to chemotherapy or radiotherapy was identified between the different CMS subtypes. Despite this, there was evidence to suggest that the use of chemotherapy in stage II disease, leads to a more deleterious result, further reinforcing that TNM staging system remains the more robust tool in determining the use of adjuvant chemotherapy. The future of the CMS system may lie in determining and predicting the use of targeted antibody treatments.

Chapter 6 showed potential incongruences in the CMS between primary CRCs and hepatic metastasis and raises the possible association of neoadjuvant chemotherapy with the switching in the subtyping of hepatic metastasis. The main limitation is that only nine patients were included in this pilot study. The implication of this and the application of neoadjuvant therapy particularly in the setting of rectal adenocarcinoma needs further investigation.

In summary, when initially published, the CMS Classification showed great promise in terms of predicting outcomes. However, findings from this thesis have shown that at its present form, the CMS classification does not outperform

the traditional TNM staging system in terms of prognosticating survival or determining and predicting response to adjuvant chemotherapy. The potential absence of CMS4 subtype further dilutes the predictive ability of the CMS classification. This together with potential incongruency of classification in the distant metastases, raises the question as to how clinically useful this classification system is. With improvements in tumour sampling techniques and enhancements in the RNA purification methods, the predictive and prognostic ability of the CMS classification system may improve. The future of individualising cancer treatment may well lie in the genetics of colorectal cancer, however, as of the current state, the CMS classification is too expensive and not clinically accurate enough to be used in day to day clinical practice.

Appendix

Table 27: Kirklin, Dockerty and Waugh modification of Dukes' Classification [12]

Kirklin, Dockerty and Waugh modification	
A	Carcinoma limited to the mucosa
B1	Carcinoma that have extended into, but not through the muscularis propria
B2	Carcinoma that have penetrated the muscularis propria
C	metastases are present in the regional lymph nodes

Table 28: Astler-Coller Modification of Dukes' Classification [13]

Astler-Coller Modification	
A	Carcinoma limited to the mucosa
B1	Lesions extending into the muscularis propria, but not penetrating it, with negative nodes
B2	Lesions penetrating the muscularis propria, with negative nodes
C1	Lesions extending into the muscularis propria, but not penetrating it, with positive nodes
C2	Lesions penetrating the muscularis propria with positive nodes

Table 29: Amsterdam II Criteria [68]

Amsterdam II Criteria
Three or more relatives with Lynch associated cancer
Two or more successive generations affected, one is first degree relative of the other two
One or more relatives is diagnosed before age of 50
Familial polyposis has been ruled out

Table 30: Revised Bethesda Guidelines[69]

Revised Bethesda Guidelines
CRC diagnosed in patient less than 50 years of age
Presence of synchronous, metachronous CRCs or HNPCC associated tumours
CRCs with MSI-H histology diagnosed in patients less than 60
CRCs diagnosed in one or more first degree relative with an HNPCC related tumour, with one cancers being diagnosed less than 60 years of age
CRCs diagnosed in two or more first or second degree relative with HNPCC related tumour, regardless of age

Table 31: Demographics and Clinico-pathological Findings of Each CMS Subtypes

		All samples		CMS1		CMS2		CMS3		CMS4	
Clinico-pathological features		**Statistics**	**P value**	**Statistics**	**P value** OR	**Statistics**	**P value** OR	**Statistics**	**P value** OR	**Statistics**	**P value** OR
Age		n=254	<0.01	n=59	<0.01	n=139	<0.01	n=40		16	<0.01
	Median	73.8		78.17		72.75		74.06		72.85	
	(range)	29-92		31-92		37-90		37-89		52-85	
Gender		n=254	<0.01	n=59	<0.01	n=139	<0.01	n=40	0.37	n=16	0.77
	Male	46.4%	118	27.1% (16)		56.1% (78)	1.61	40% (16)		50% (8)	
	Female	53.5%	136	72.9% (43)	2.95	43.9% (61)		60% (24)	1.15	50% (8)	1.17
Site		n=254	<0.01	n=59	<0.01	n=139	<0.01	n=40	0.29	n=16	0.05
	Right colon	43.7% (111)		79.7% (47)	2.42	28.1% (39)		55% (22)		18.8% (3)	
	Left colon	37.4% (95)		18.6% (11)		46.8% (65)	1.92	30% (12)		43.8% (7)	2.42
	Rectum	18.9% (48)		1.7% (1)		25.2% (35)		15% (6)		37.5% (6)	
Stage		n=254	<0.01	n=59	0.07	n=139	0.54	n=40	0.06	n=16	0.03
	I	18.9% (48)		16.9% (10)		17.3% (24)		30% (12)		12.5% (2)	
	II	42.9% (109)		55.9% (33)		44.6% (62)		27.5% (11)		18.8% (3)	
	III	31.9.3% (81)		25.4% (15)		30.2% (42)		40% (16)		50% (8)	
	IV	6.3% (16)		1.7% (1)		7.9% (11)		2.5% (1)		18.8% (3)	
T Staging		n=254	0.04	n=59	0.51	n=139	0.06	n=40	<0.01	n=16	0.86
	1	3.1% (8)		3.4% (2)		0.7% (1)		12.5% (5)		0% (0)	
	2	19.7% (50)		15.3% (9)		21.6% (30)		20% (8)		18.8% (3)	
	3	62.2% (158)		61% (36)		64.7% (90)		52.5% (21)		68.8%(11)	
	4	15% (38)		20.3% (12)		12.8% (18)		15% (6)		12.5% (2)	
N Staging		n=254	0.02	n=59	0.03	n=139	0.92	n=40	0.55	n=16	<0.01
	0	62.6% (159)		76.3% (45)	1.75	61.9% (86)		57.5% (23)		31.2% (5)	
	1	25.6% (65)		18.6% (11)		26.6% (65)		32.5% (13)		25% (4)	
	2	11.8% (30)		5.1% (3)		11.5% (30)		10% (4)		43.8% (7)	

M staging		n=254	0.04	n=59	0.10	n=139	0.24	n=40	n=0.28	n=16	0.03
	0	93.7% (238)		98.3% (58)		92.1% (128)		97.5% (39)		81.2% (13)	
	1	6.3% (16)		1.7% (1)	0.93	7.9% (11)	1.03	2.5% (1)	2.8	18.8% (3)	3.44
Poorly differentiated		n=254	<0.01	n=59	<0.01	n=139	<0.01	n=40	0.24	n=16	0.48
	No	80.7% (205)		50.8% (30)		90.6% (126)	1.39	87.5% (35)		87.5% (14)	
	Yes	19.3% (49)		49.2% (29)	4.78	9.4% (13)		12.5% (5)	0.91	12.5% (2)	1.58
Mucinous		n=254	<0.01	n=59	<0.01	n=139	<0.01	n=40	0.07	n=16	0.71
	No	92% (229)		79.7% (47)		97.1% (135)	6.34	82.5% (33)	1.11	87.5% (14)	1.33
	Yes	9.8% (25)		20.3% (12)	3.57	2.9% (4)		17.5% (7)		12.5% (2)	
signet ring		n=254	0.34	n=59	0.37	n=139	0.12	n=40	0.18	n=16	0.71
	No	99.2% (252)		98.3% (58)	1.01	100% (139)	0.98	97.5% (39)	1.02	100% (16)	0.99
	yes	0.8% (2)		1.7% (1)		0%		2.5% (1)		0%	
Lymphovascular invasion		n=254	0.97	n=59	0.91	n=139	0.66	n=40	0.70	n=16	0.91
	No	70.1% (178)		69.5% (41)	1.01	71.2% (99)	0.96	67.5% (27)	1.04	68.8% (11)	1.02
	Yes	29.9% (76)		30.5% (18)		28.8% (40)		32.5% (13)		32.7% (5)	
perineural invasion		n=254	0.59	n=59	0.54	n=139	0.62	n=40	0.48	n=16	0.28
	No	89.4% (227)		91.5% (54)	0.96	88.5% (123)	1.02	92.5% (37)	0.96	81.2% (13)	1.11
	Yes	10.6% (27)		8.5% (5)		11.5% (16)		7.5% (3)		18.8% (3)	
isolated extramural deposit		n=254	0.03	n=59	0.74	n=139	0.50	n=40	0.19	n=16	<0.01
	No	92.5% (235)		91.5% (54)	1.01	93.5% (130)	0.97	97.5% (39)	0.94	75% (12)	
	Yes	7.5% (19)		8.5% (5)		6.5% (9)		2.5%(1)		25% (4)	3.97
extravenous invasion		n=254	0.35	n=59	0.70	n=139	0.61	n=40	0.23	n=16	0.16

	No	86.6% (220)		88.1% (52)	0.98	85.6% (119)	1.02	92.5% (37)	0.92	75% (12)	1.17
	Yes	13.4% (34)		11.9% (7)		14.4% (20)		7.5% (3)		25% (4)	
Adjuvant chemotherapy		n=254	0.01	n=59	0.03	n=139	0.17	n=40	0.94	n=16	0.47
	no	67% (205)		81% (48)		66.9% (93)		70% (28)		62.5% (10)	
	yes	33% (101)		18.6% (11)	1.76	33.1% (46)		30% (12)		37.5% (6)	
MSI		n=32	<0.01	n=10	<0.01	n=12	<0.01	n=9	0.76	n=1	0.43
	No	62.5% (20)		10% (1)		100%	0.4	66.7% (6)	0.77	100%	0.95
	Yes	37.5% (12)		90% (9)	57	0%		33.3% (3)		0%	
Local Recurrance		n=254	0.55	n=59		n=139		n=40		n=16	
		10 (3.9%)		3.9% (2)		3.6% (5)		7% (3)		0% (0)	
Subsequent distant metastasis		n=254	0.19	n=59		n=139		n=40		n=16	
		45 (17.7%)		11.9% (7)		20.1% (28)		12.5% (5)		31.2% (5)	

Cells containing red numbers denote significant clinical or histological findings for that subtype

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